

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

United States Patent and Trademark
Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 19 August 1998 (19.08.98)	
International application No. PCT/US97/20873	Applicant's or agent's file reference GC369-2-PCT
International filing date (day/month/year) 13 November 1997 (13.11.97)	Priority date (day/month/year) 13 November 1996 (13.11.96)
Applicant DUNN-COLEMAN, Nigel, S. et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

12 June 1998 (12.06.98)

☐ in a notice effecting later election filed with the International Bureau on:2. The election ☒ was☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer P. Regis
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

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PATENT COOPERATION TREATY

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference GC369-2-PCT	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/US 97/ 20873	International filing date (day/month/year) 13/11/1997	(Earliest) Priority Date (day/month/year) 13/11/1996
Applicant GENENCOR INTERNATIONAL INC, et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. ☐ Certain claims were found unsearchable (see Box I).

2. ☐ Unity of invention is lacking (see Box II).

3. ☒ The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing

☒ filed with the international application.

☐ furnished by the applicant separately from the international application,

☐ but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.

☐ Transcribed by this Authority

4. With regard to the title, ☒ the text is approved as submitted by the applicant

☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

☒ the text is approved as submitted by the applicant

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is:

Figure No. ☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/20873

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/53 C12N15/55 C12N15/60 C12P7/18 C12N9/04
C12N9/16 C12N9/88

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X, L	WO 96 35796 A (DU PONT ; GENENCOR INT (US); LAFFEND LISA ANNE (US); NAGARAJAN VASA) 14 November 1996 see the whole document see abstract see examples 2-5 see examples 22, 23 see page 62, paragraph 2	1-40
P, A	WO 96 35795 A (DU PONT ; NAGARAJAN VASANTHA (US); NAKAMURA CHARLES EDWIN (US)) 14 November 1996 see abstract see page 9 see examples 1-3	1-13, 15-40



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

21 April 1998

Date of mailing of the international search report

08/05/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Lejeune, R

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 97/20873

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>TOBIMATSU T. ET AL.: "Molecular cloning, sequencing, and expression of the genes encoding adenosylcobalamin-dependent diol dehydrase of <i>Klebsiella oxytoca</i>." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 13, 31 March 1995, pages 7142-7148, XP002062740 cited in the application see page 7147, column 2, paragraph 2 see page 7145, column 1, line 1 - page 7146, column 1, line 4 see abstract</p> <p>---</p>	1,5,7,8, 10,11
A	<p>TOBIMATSU T. ET AL.: "Cloning, sequencing and high level expression of the genes encoding adenosylcobalamin-dependent glycerol dehydrase of <i>Klebsiella pneumoniae</i>." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 37, 13 September 1996, pages 22352-22357, XP002057923 see abstract see page 22356, column 1, line 2 - line 5 see page 22356, column 2, paragraph 4 - page 22357, column 1, paragraph 1</p> <p>---</p>	1,4,6,8, 10,11,15
A	<p>TONG I.T. ET AL.: "1,3-propanediol production by <i>Escherichia coli</i> expressing genes from the <i>Klebsiella pneumoniae</i> dha regulon." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 57, 1991, pages 3541-3546, XP002062741 see abstract</p> <p>---</p>	1-4,6,8, 10,11
A	<p>SEYFRIED M. ET AL.: "Cloning, sequencing, and overexpression of the genes encoding coenzyme B12-dependent glycerol dehydratase of <i>Citrobacter freundii</i>." JOURNAL OF BACTERIOLOGY, vol. 178, no. 19, October 1996, pages 5793-5796, XP002062742 cited in the application see page 5794; figure 1 see page 5793, column 2, paragraph 5 see abstract</p> <p>-----</p>	1-4,6

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/20873

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9635796 A	14-11-96	US 5686276 A AU 5678996 A EP 0826057 A	11-11-97 29-11-96 04-03-98
WO 9635795 A	14-11-96	US 5633362 A AU 5722996 A EP 0827543 A	27-05-97 29-11-96 11-03-98

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PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: DEBRA J. GLAISTER
GENENCOR INTERNATIONAL, INC.
925 PAGE MILL ROAD
PALO ALTO, CALIFORNIA 94304-1013

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

GC364-2-PCT

Date of Mailing (day/month/year) **11 JAN 1999**

Applicant's or agent's file reference

GC364-2-PCT

IMPORTANT NOTIFICATION

International application No.

PCT/US97/20873

International filing date (day/month/year)

13 NOVEMBER 1997

Priority Date (day/month/year)

13 NOVEMBER 1996

Applicant

GENENCOR INTERNATIONAL, INC.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.
4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

NASHAAT T. NASHED

Telephone No. (703) 308-0196

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PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference GC396-2-PCT	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US97/20873	International filing date (day/month/year) 13 NOVEMBER 1997	Priority date (day/month/year) 13 NOVEMBER 1996
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant GENENCOR INTERNATIONAL, INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of <u>4</u> sheets. <input checked="" type="checkbox"/> This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT). These annexes consist of a total of <u>4</u> sheets.
3. This report contains indications relating to the following items: <ul style="list-style-type: none"> I <input checked="" type="checkbox"/> Basis of the report II <input type="checkbox"/> Priority III <input type="checkbox"/> Non-establishment of report with regard to novelty, inventive step or industrial applicability IV <input type="checkbox"/> Lack of unity of invention V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement VI <input type="checkbox"/> Certain documents cited VII <input type="checkbox"/> Certain defects in the international application VIII <input type="checkbox"/> Certain observations on the international application

Date of submission of the demand 12 JUNE 1998	Date of completion of this report 03 NOVEMBER 1998
Name and mailing address of the IPEA-US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer <div style="text-align: center;"> NASHAAT T. NASHED </div>
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US97/20873

I. Basis of the report

1. This report has been drawn on the basis of (*Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments*):

☐ the international application as originally filed.

☒ the description, pages 1-100, as originally filed.

pages None, filed with the demand.

pages NONE, filed with the letter of _____.

pages _____, filed with the letter of _____.

☒ the claims, Nos. None, as originally filed.

Nos. None, as amended under Article 19.

Nos. 1-40, filed with the demand.

Nos. NONE, filed with the letter of _____.

Nos. _____, filed with the letter of _____.

☒ the drawings, sheets/fig 1-27, as originally filed.

sheets/fig None, filed with the demand.

sheets/fig NONE, filed with the letter of _____.

sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

☒ the description, pages None.

☒ the claims, Nos. None.

☒ the drawings, sheets/fig None.

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box. Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US97/20873

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. STATEMENT**

Novelty (N)	Claims <u>1-40</u>	YES
	Claims <u>None</u>	NO
Inventive Step (IS)	Claims <u>1-40</u>	YES
	Claims <u>None</u>	NO
Industrial Applicability (IA)	Claims <u>1-40</u>	YES
	Claims <u>None</u>	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-40 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest the coexpression of the nucleic acid sequence encoding protein X and diol or glycerol dehydratase in a microorganism to enhance the stability and the activity of the dehydratase *in vivo*. The transformed microorganism is more efficient in producing 1,3-propanediol.

----- NEW CITATIONS -----
NONE

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US97/20873

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below: **IPC(6): C12P 7/18; C12N 1/14, 1/20, 9/96 and US Cl.: 435/158, 188, 252.3, 252.31, 252.33, 252.34, 252.35, 254.11, 254.2, 254.21, 254.22, 254.23, 254.3**

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101

WHAT IS CLAIMED IS:

1. An improved method for the production of 1,3-propanediol from a microorganism comprising the steps of:

a) obtaining a recombinant microorganism capable of producing 1,3-propanediol, said microorganism comprising at least one nucleic acid encoding a dehydratase activity and a nucleic acid encoding protein X; and

b) culturing the recombinant microorganism in the presence of at least one carbon source capable of being converted to 1,3 propanediol in said transformed microorganism and under conditions suitable for the production of 1,3 propanediol wherein the carbon source is selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and a one carbon substrate.

2. The method of Claim 1 wherein said recombinant microorganism comprises at least one nucleic acid encoding a protein selected from the group consisting of protein 1, protein 2 and protein 3.

3. The method of Claim 1 further comprising the step of recovering the 1,3 propanediol.

4. The method of Claim 1 wherein the nucleic acid encoding protein X is isolated from a glycerol dehydratase gene cluster.

5. The method of Claim 1 wherein the nucleic acid encoding protein X is isolated from a diol dehydratase gene cluster.

6. The method of Claim 4 wherein the glycerol dehydratase gene cluster is from an organism selected from the genera consisting of Klebsiella and Citrobactor.

7. The method of Claim 5 wherein the diol dehydratase gene cluster is from an organism selected from the genera consisting of Klebsiella, Clostridium and Salmonella.

8. The method of Claim 1 wherein the nucleic acid encoding a dehydratase activity is heterologous to the organism.

9. The method of Claim 1 wherein the nucleic acid encoding a dehydratase activity is homologous to the organism.

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10. The method of Claim 1 wherein the recombinant microorganism is selected from the group of genera consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*.

11. The method of Claim 10 wherein the microorganism is selected from the group consisting of *E. coli* and *Klebsiella* spp.

12. The method of Claim 1 wherein the nucleic acid encoding protein X is stably maintained in the host genome.

13. The method of Claim 2 wherein at least one nucleic acid encoding a protein selected from protein 1, protein 2 and protein 3 is stably maintained in the host genome.

14. The method of Claim 1 wherein the carbon source is glucose.

15. The method of Claim 1 wherein the nucleic acid encoding protein X has the sequence as shown in SEQ ID NO: 59.

16. The method of Claim 2 wherein protein 1 has the sequence as shown in SEQ ID NO: 60 or SEQ ID NO: 61.

17. The method of Claim 2 wherein protein 2 has the sequence as shown in SEQ ID NO: 62 or SEQ ID NO: 63.

18. The method of Claim 2 wherein protein 3 has the sequence as shown in SEQ ID NO: 64 or SEQ ID NO: 65.

19. A recombinant microorganism capable of producing 1,3-propanediol from a carbon source said recombinant microorganism comprising a) at least one nucleic acid encoding a dehydratase activity; b) at least one nucleic acid encoding a glycerol-3-phosphatase; and c) at least one nucleic acid encoding protein X.

20. The recombinant microorganism of Claim 19 further comprising d) at least one nucleic acid encoding a protein selected from the group consisting of protein 1, protein 2 and protein 3.

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21. The recombinant microorganism of Claim 19 selected from the group consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*.

22. The recombinant microorganism of Claim 19 wherein the nucleic acid encoding protein X is isolated from a glycerol dehydratase gene cluster.

23. The recombinant microorganism of Claim 19 wherein the nucleic acid encoding protein X is isolated from a diol dehydratase gene cluster.

24. The recombinant microorganism of Claim 22 wherein the glycerol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella* and *Citrobacter*.

25. The recombinant microorganism of Claim 23 wherein the diol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella*, *Clostridium* and *Salmonella*.

26. The recombinant microorganism of Claim 19 wherein said dehydratase activity is heterologous to said microorganism.

27. The recombinant microorganism of Claim 19 wherein said dehydratase activity is homologous to said microorganism.

28. The recombinant microorganism of Claim 19 wherein the nucleic acid encoding protein X has the sequence as shown in SEQ ID NO: 59.

29. The recombinant microorganism of Claim 20 wherein protein 1 has the sequence as shown in SEQ ID NO: 60 or SEQ ID NO: 61.

30. The recombinant microorganism of Claim 20 wherein protein 2 has the sequence as shown in SEQ ID NO: 62 or SEQ ID NO: 63.

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31. The recombinant of Claim 20 wherein protein 3 has the sequence as shown in SEQ ID: 64 or SEQ ID NO: 65.

32. A method for extending the half-life of dehydratase activity in a transformed microorganism capable of producing 1,3-propanediol and containing at least one nucleic acid encoding a dehydratase activity, comprising the step of introducing a nucleic acid encoding protein X into said microorganism and culturing under conditions suitable for production of 1,3-propanediol.

33. The method of Claim 32 wherein the nucleic acid encoding the dehydratase activity is heterologous to said microorganism.

34. The method of Claim 32 wherein the nucleic acid encoding the dehydratase activity is homologous to said microorganism.

35. The method of Claim 32 wherein the nucleic acid encoding protein X is isolated from a glycerol dehydratase gene cluster.

36. The method of Claim 32 wherein the nucleic acid encoding protein X is isolated from a diol dehydratase gene cluster.

37. The method of Claim 35 wherein the glycerol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella* and *Citrobacter*.

38. The method of Claim 34 wherein the diol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella*, *Clostridium* and *Salmonella*.

39. The method of Claim 32 wherein the microorganism is selected from the group consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*.

40. The method of Claim 32 further comprising the step of introducing at least one nucleic acid encoding protein 1, protein 2 or protein 3 into said microorganism.

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WHAT IS CLAIMED IS:

1. An improved method for the production of 1,3-propanediol from an organism capable of producing 1,3-propanediol, said organism comprising at least one gene encoding a dehydratase activity, the method comprising the steps of:
 - (a) introducing a gene encoding protein X into the organism to create a transformed organism; and
 - (b) culturing the transformed organism in the presence of at least one carbon source capable of being converted to 1,3 propanediol in said transformed host organism and under conditions suitable for the production of 1,3 propanediol wherein the carbon source is selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and a one carbon substrate.
2. The method of Claim 1 further comprising the step of introducing at least one gene encoding a protein selected from the group consisting of protein 1, protein 2 and protein 3 into the organism.
3. The method of Claim 1 further comprising the step of recovering the 1,3 propanediol.
4. The method of Claim 1 wherein the gene encoding protein X is isolated from a glycerol dehydratase gene cluster.
5. The method of Claim 1 wherein the gene encoding protein X is isolated from a diol dehydratase gene cluster.
6. The method of Claim 4 wherein the glycerol dehydratase gene cluster is from an organism selected from the genera consisting of Klebsiella and Citrobactor.
7. The method of Claim 5 wherein the diol dehydratase gene cluster is from an organism selected from the genera consisting of Klebsiella, Clostridium and Salmonella.
8. The method of Claim 1 wherein the gene encoding a dehydratase activity is heterologous to the organism.
9. The method of Claim 1 wherein the gene encoding a dehydratase activity is homologous to the organism.

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10. The method of Claim 1 wherein the organism is selected from the group of genera consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*.
11. The method of Claim 10 wherein the organism is selected from the group consisting of *E.coli* and *Klebsiella spp.*
12. The method of Claim 1 wherein the gene encoding protein X is stably maintained in the host genome.
13. The method of Claim 2 wherein at least one gene encoding a protein selected from protein 1, protein 2 and protein 3 is stably maintained in the host genome.
14. The method of Claim 1 wherein the carbon source is glucose.
15. The method of Claim 1 wherein the gene encoding protein X has the sequence as shown in SEQ ID NO: 59.
16. The method of Claim 2 wherein protein 1 has the sequence as shown in SEQ ID NO: 60 or SEQ ID NO: 61.
17. The method of Claim 2 wherein protein 2 has the sequence as shown in SEQ ID NO: 62 or SEQ ID NO: 63.
18. The method of Claim 2 wherein protein 3 has the sequence as shown in SEQ ID NO: 64 or SEQ ID NO: 65.
19. A recombinant microorganism capable of producing 1,3-propanediol from a carbon source said recombinant microorganism comprising a) at least one gene encoding a dehydratase activity; b) at least one gene encoding a glycerol-3-phosphatase; and c) at least one gene encoding protein X.
20. The recombinant microorganism of Claim 19 further comprising d) at least one gene encoding a protein selected from the group consisting of protein 1, protein 2 and protein 3.

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21. The recombinant microorganism of Claim 19 selected from the group consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*.
22. The recombinant microorganism of Claim 19 wherein the gene encoding protein X is isolated from a glycerol dehydratase gene cluster.
23. The recombinant microorganism of Claim 19 wherein the gene encoding protein X is isolated from a diol dehydratase gene cluster.
24. The recombinant microorganism of Claim 22 wherein the glycerol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella* and *Citrobacter*.
25. The recombinant microorganism of Claim 23 wherein the diol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella*, *Clostridium* and *Salmonella*.
26. The recombinant microorganism of Claim 19 wherein said dehydratase activity is heterologous to said microorganism.
27. The recombinant microorganism of Claim 19 wherein said dehydratase activity is homologous to said microorganism.
28. The recombinant microorganism of Claim 19 wherein the gene encoding protein X has the sequence as shown in SEQ ID NO: 59.
29. The recombinant microorganism of Claim 20 wherein protein 1 has the sequence as shown in SEQ ID NO: 60 or SEQ ID NO: 61.
30. The method of Claim 20 wherein protein 2 has the sequence as shown in SEQ ID NO: 62 or SEQ ID NO: 63.

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31. The method of Claim 20 wherein protein 3 has the sequence as shown in SEQ ID: 64 or SEQ ID NO: 65.

32. A method for extending the halflife of dehydratase activity in a microorganism capable of producing 1,3-propanediol and containing at least one gene encoding a dehydratase activity, comprising the step of introducing a gene encoding protein X into said microorganism and culturing under conditions suitable for production of 1,3-propanediol.

33. The method of Claim 32 wherein the gene encoding the dehydratase activity is heterologous to said microorganism.

34. The method of Claim 32 wherein the gene encoding the dehydratase activity is homologous to said microorganism.

35. The microorganism of Claim 32 wherein the gene encoding protein X is isolated from a glycerol dehydratase gene cluster.

36. The microorganism of Claim 32 wherein the gene encoding protein X is isolated from a diol dehydratase gene cluster.

37. The microorganism of Claim 35 wherein the glycerol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella* and *Citrobacter*.

38. The microorganism of Claim 34 wherein the diol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella*, *Clostridium* and *Salmonella*.

39. The method of Claim 32 wherein the microorganism is selected from the group consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*.

40. The method of Claim 32 further comprising the step of introducing a gene encoding at least one of protein 1, protein 2 and protein 3 into said microorganism.

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Amended

WHAT IS CLAIMED IS:

1. An improved method for the production of 1,3-propanediol from a microorganism comprising the steps of:
 - a) obtaining a recombinant microorganism capable of producing 1,3-propanediol, said microorganism comprising at least one nucleic acid encoding a dehydratase activity and a nucleic acid encoding protein X; and
 - b) culturing the recombinant microorganism in the presence of at least one carbon source capable of being converted to 1,3 propanediol in said transformed microorganism and under conditions suitable for the production of 1,3 propanediol wherein the carbon source is selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and a one carbon substrate.
2. The method of Claim 1 wherein said recombinant microorganism comprises at least one nucleic acid encoding a protein selected from the group consisting of protein 1, protein 2 and protein 3.
3. The method of Claim 1 further comprising the step of recovering the 1,3 propanediol.
4. The method of Claim 1 wherein the nucleic acid encoding protein X is isolated from a glycerol dehydratase gene cluster.
5. The method of Claim 1 wherein the nucleic acid encoding protein X is isolated from a diol dehydratase gene cluster.
6. The method of Claim 4 wherein the glycerol dehydratase gene cluster is from an organism selected from the genera consisting of Klebsiella and Citrobactor.
7. The method of Claim 5 wherein the diol dehydratase gene cluster is from an organism selected from the genera consisting of Klebsiella, Clostridium and Salmonella.
8. The method of Claim 1 wherein the nucleic acid encoding a dehydratase activity is heterologous to the organism.
9. The method of Claim 1 wherein the nucleic acid encoding a dehydratase activity is homologous to the organism.

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10. The method of Claim 1 wherein the recombinant microorganism is selected from the group of genera consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*.

11. The method of Claim 10 wherein the microorganism is selected from the group consisting of *E.coli* and *Klebsiella* spp.

12. The method of Claim 1 wherein the nucleic acid encoding protein X is stably maintained in the host genome.

13. The method of Claim 2 wherein at least one nucleic acid encoding a protein selected from protein 1, protein 2 and protein 3 is stably maintained in the host genome.

14. The method of Claim 1 wherein the carbon source is glucose.

15. The method of Claim 1 wherein the nucleic acid encoding protein X has the sequence as shown in SEQ ID NO: 59.

16. The method of Claim 2 wherein protein 1 has the sequence as shown in SEQ ID NO: 60 or SEQ ID NO: 61.

17. The method of Claim 2 wherein protein 2 has the sequence as shown in SEQ ID NO: 62 or SEQ ID NO: 63.

18. The method of Claim 2 wherein protein 3 has the sequence as shown in SEQ ID NO: 64 or SEQ ID NO: 65.

19. A recombinant microorganism capable of producing 1,3-propanediol from a carbon source said recombinant microorganism comprising a) at least one nucleic acid encoding a dehydratase activity; b) at least one nucleic acid encoding a glycerol-3-phosphatase; and c) at least one nucleic acid encoding protein X.

20. The recombinant microorganism of Claim 19 further comprising d) at least one nucleic acid encoding a protein selected from the group consisting of protein 1, protein 2 and protein 3.

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21. The recombinant microorganism of Claim 19 selected from the group consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*.
22. The recombinant microorganism of Claim 19 wherein the nucleic acid encoding protein X is isolated from a glycerol dehydratase gene cluster.
23. The recombinant microorganism of Claim 19 wherein the nucleic acid encoding protein X is isolated from a diol dehydratase gene cluster.
24. The recombinant microorganism of Claim 22 wherein the glycerol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella* and *Citrobacter*.
25. The recombinant microorganism of Claim 23 wherein the diol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella*, *Clostridium* and *Salmonella*.
26. The recombinant microorganism of Claim 19 wherein said dehydratase activity is heterologous to said microorganism.
27. The recombinant microorganism of Claim 19 wherein said dehydratase activity is homologous to said microorganism.
28. The recombinant microorganism of Claim 19 wherein the nucleic acid encoding protein X has the sequence as shown in SEQ ID NO: 59.
29. The recombinant microorganism of Claim 20 wherein protein 1 has the sequence as shown in SEQ ID NO: 60 or SEQ ID NO: 61.
30. The recombinant microorganism of Claim 20 wherein protein 2 has the sequence as shown in SEQ ID NO: 62 or SEQ ID NO: 63.

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31. The recombinant of Claim 20 wherein protein 3 has the sequence as shown in SEQ ID: 64 or SEQ ID NO: 65.

32. A method for extending the half-life of dehydratase activity in a transformed microorganism capable of producing 1,3-propanediol and containing at least one nucleic acid encoding a dehydratase activity, comprising the step of introducing a nucleic acid encoding protein X into said microorganism and culturing under conditions suitable for production of 1,3-propanediol.

33. The method of Claim 32 wherein the nucleic acid encoding the dehydratase activity is heterologous to said microorganism.

34. The method of Claim 32 wherein the nucleic acid encoding the dehydratase activity is homologous to said microorganism.

35. The method of Claim 32 wherein the nucleic acid encoding protein X is isolated from a glycerol dehydratase gene cluster.

36. The method of Claim 32 wherein the nucleic acid encoding protein X is isolated from a diol dehydratase gene cluster.

37. The method of Claim 35 wherein the glycerol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella* and *Citrobacter*.

38. The method of Claim 34 wherein the diol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella*, *Clostridium* and *Salmonella*.

39. The method of Claim 32 wherein the microorganism is selected from the group consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*.

40. The method of Claim 32 further comprising the step of introducing at least one nucleic acid encoding protein 1, protein 2 or protein 3 into said microorganism.

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PCT

FEE CALCULATION SHEET

Annex to the Demand for International Preliminary Examination

International Application No. PCT/US97/20873	For International Preliminary Examining Authority use only
Applicant's or agent's file reference GC369-2-PCT	Date stamp of the IPEA
Applicant GENENCOR INTERNATIONAL, INC.	
Calculation of prescribed fees	
1. Preliminary examination fee	\$ 162.00 P
2. Handling fee (Applicants from certain States are entitled to a reduction of 75% of the handling fee. Where the applicant is (or applicants are) so entitled, the amount to be entered at H is 25% of the handling fee.)	\$ 750.00 H
3. Total of prescribed fees Add the amounts entered at P and H and enter total in the TOTAL box	<div style="border: 1px solid black; padding: 5px; display: inline-block;"> \$ 912.00 Total </div>
Mode of Payment	
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <input checked="" type="checkbox"/> authorization to charge deposit account with the IPEA (see below) </div> <div style="width: 45%;"> <input type="checkbox"/> cash </div> </div>	
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <input type="checkbox"/> cheque </div> <div style="width: 45%;"> <input type="checkbox"/> revenue stamps </div> </div>	
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <input type="checkbox"/> postal money order </div> <div style="width: 45%;"> <input type="checkbox"/> coupons </div> </div>	
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <input type="checkbox"/> bank draft </div> <div style="width: 45%;"> <input type="checkbox"/> other (specify) </div> </div>	
Deposit Account Authorization (this mode of payment may not be available at all IPEAs)	
The IPEA/US <input checked="" type="checkbox"/> is hereby authorized to charge the total fees indicated above to my deposit account.	
<input checked="" type="checkbox"/> (this check-box may be marked only if the conditions for deposit accounts of the IPEA so permit) is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.	
07-1048	12 June 1998
Deposit Account Number	Date (day/month/year)
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> </div> <div style="width: 45%;"> Signature Printed Name Debra J. GLAISTER </div> </div>	

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Sent to Dep't
9-8-98

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN THE UNITED STATES RECEIVING OFFICE

In re International Patent Application of

GENENCOR INTERNATIONAL, INC.

Serial No. PCT/US97/20873

Filed: 13 November 1997

For: Method for Recombinant
Production of 1,3 Propanediol

TRANSMITTAL OF
CHAPTER 2 DEMAND

EL070473268US

I hereby certify that this correspondence is being deposited with the
United States Postal Service as EXPRESS MAIL, postage prepaid,
addressed to: Assistant Commissioner of Patents, Washington, D.C.
20231, Box PCT, on June 12, 1998.

June 12, 1998

By:

Anke E. Luttrell

BOX PCT
Honorable Commissioner
of Patents and Trademarks
Washington, D.C. 20231

Dear Sir:

Attached, for filing in the above-identified International Patent Application, is the
Chapter 2 Demand for Preliminary Examination, including a copy of our letter and
enclosures, dated 12 June 1998, to WIPO in Switzerland submitting amended claims under
Article 19.

Please charge the requisite fees to our Deposit Account 07-1048. This
correspondence is transmitted in triplicate for that purpose.

Respectfully submitted,

Date:

June 12, 1998

By:

Debra J. Glaister

Debra J. GLAISTER
Reg. No. 33,888

Genencor International, Inc.
925 Page Mill Road
Palo Alto, California 94304-1013

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The demand must be filed directly with the competent International Preliminary Examining Authority, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below:

IPEA/US

PCT DEMAND

CHAPTER II

under Article 31 of the Patent Cooperation Treaty:

The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty.

For International Preliminary Examining Authority use only

Identification of IPEA

Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference

GC369-2-PCT

International application No.

PCT/US97/20873

International filing date (day/month/year)

13 November 1997 - 13.11.97

(Earliest) Priority date (day/month/year)

13 November 1996 - 13.11.96

Title of invention

Method for the Recombinant Production of 1,3-Propanediol

Box No. II - APPLICANT(S)

Name and address: (Family name followed by given name, for legal entity, full official designation. The address must include postal code and name of country.)

**GENENCOR INTERNATIONAL, INC.
4 CAMBRIDGE PLACE
1870 SOUTH WINTON ROAD
ROCHESTER, NEW YORK 14618
UNITED STATES OF AMERICA**

Telephone No. **650-846-7620**

Facsimile No. **650-845-6504**

Teleprinter No.

State (i.e. country) of nationality:

US

State (i.e. country) of residence:

US

Name and address: (Family name followed by given name, for legal entity, full official designation. The address must include postal code and name of country.)

**DUNN-COLEMAN, Nigel S.
142 Johnson Avenue
Los Gatos, California 95032
United States of America**

State (i.e. country) of nationality:

GB

State (i.e. country) of residence:

US

Name and address: (Family name followed by given name, for legal entity, full official designation. The address must include postal code and name of country.)

**DIAZ-TORRES, Maria
58 North El Camino Real
San Mateo, California 94401
United States of America**

State (i.e. country) of nationality:

ES

State (i.e. country) of residence:

US

☒ Further applicants are indicated on another continuation sheet.

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Continuation of Box No. II APPLICANT(S)

Name and address: (Family name followed by given name; for legal entity, full official designation. The address must include postal code and name of country.)

CHASE, Matthew W.
2211-Hastings Drive
Belmont, California 91402
United States of America

State (i.e. country) of nationality:

US

State (i.e. country) of residence:

US

Name and address: (Family name followed by given name; for legal entity, full official designation. The address must include postal code and name of country.)

TRIMBUR, Donald
349 Orchard Avenue
Redwood City, California 94601
United States of America

State (i.e. country) of nationality:

US

State (i.e. country) of residence:

US

Name and address: (Family name followed by given name; for legal entity, full official designation. The address must include postal code and name of country.)

State (i.e. country) of nationality:

State (i.e. country) of residence:

Name and address: (Family name followed by given name; for legal entity, full official designation. The address must include postal code and name of country.)

State (i.e. country) of nationality:

State (i.e. country) of residence:

☐ Further applicants are indicated on another continuation sheet.

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Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCEThe following person is ☒ agent ☐ common representativeand ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.Name and address (Family name followed by given name; for a legal entity, full official designation.
The address must include postal code and name of country.)GLAISTER, Debra J.
GENENCOR INTERNATIONAL, INC.
925 Page Mill Road
Palo Alto, California 94304-1013
United States of America

Telephone No.

650-846-7620

Facsimile No.

650-845-6504

Teleprinter No.:

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.**Box No. IV STATEMENT CONCERNING AMENDMENTS**

The applicant wishes the International Preliminary Examining Authority*

(i) ☐ to start the international preliminary examination on the basis of the international application as originally filed.(ii) ☐ to take into account the amendments under Article 34 of☐ the description (amendments attached).☐ the claims (amendments attached).☐ the drawings (amendments attached).(iii) ☒ to start the international preliminary examination on the basis of the international application as originally filed taking into account any amendments of the claims under Article 19 filed with the International Bureau (a copy is attached) as well as taking into account the complete sequence listings submitted to the European Patent Office under date of 02 April 1998.(iv) ☐ to disregard any amendments of the claims made under Article 19 and to consider them as reversed.(v) ☐ to postpone the start of the international preliminary examination until the expiration of 20 months from the priority date unless that Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). (This check-box may be marked only where the time limit under Article 19 has not yet expired.)

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Box No. V ELECTION OF STATES☒ The applicant hereby elects all eligible States (that is, all States which have been designated and which are bound by Chapter II of the PCT) except

(If the applicant does not wish to elect certain eligible States, the name(s) or country code(s) of those States must be indicated above.)

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Box N . VI CHECK LIST

The demand is accompanied by the following documents for the purposes of international preliminary examination:

For International Preliminary Examination Authority use only

1. amendments under Article 34

description	sheets
claims	sheets
drawings	sheets

2. letter accompanying amendments under Article 34

sheets

3. copy of amendments under Art. 19

4

sheets

4. copy of statement under Art. 19

1

sheets

5. other (specify): 1p. letter of 02 April 1998 to the European Patent Office and 52 sheets of sequence listings

53

sheets

received

not received

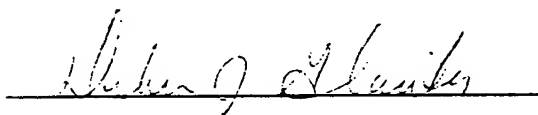
☐☐☐☐☐☐☐☐☐☐☐☐☐☐

The demand is also accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney4. ☒ fee calculation sheet2. ☐ copy of general power of attorney5. ☐ other (specify)3. ☐ statement explaining lack of signature

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).



Debra J. GLAISTER

For International Preliminary Examining Authority use only

1. Date of actual receipt of Demand

2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):

3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below does not apply ☐ The applicant has been informed accordingly.4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 85.

For International Bureau use only

Demand received from IPEA on:

Form PCT/IPEA/401 (last Sheet)

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

REC'D 19 JAN 1999
 IPO PCT

Applicant's or agent's file reference GC396-2-PCT	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US97/20873	International filing date (day/month/year) 13 NOVEMBER 1997	Priority date (day/month/year) 13 NOVEMBER 1996
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant GENENCOR INTERNATIONAL, INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand 12 JUNE 1998	Date of completion of this report 03 NOVEMBER 1998
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer NASHAAT T. NASHED
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US97/20873

I. Basis of the report

1. This report has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments):*

- ☐ the international application as originally filed.
- ☒ the description, pages 1-100 , as originally filed.
pages None , filed with the demand.
pages NONE , filed with the letter of _____
pages _____ , filed with the letter of _____
- ☒ the claims, Nos. None , as originally filed.
Nos. None , as amended under Article 19.
Nos. 1-40 , filed with the demand.
Nos. NONE , filed with the letter of _____
Nos. _____ , filed with the letter of _____
- ☒ the drawings, sheets/fig 1-27 , as originally filed.
sheets/fig None , filed with the demand.
sheets/fig NONE , filed with the letter of _____
sheets/fig _____ , filed with the letter of _____

2. The amendments have resulted in the cancellation of:

- ☒ the description, pages None
- ☒ the claims, Nos. None
- ☒ the drawings, sheets/fig None

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US97/20873

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. STATEMENT**

Novelty (N)

Claims	<u>1-40</u>	YES
Claims	<u>None</u>	NO

Inventive Step (IS)

Claims	<u>1-40</u>	YES
Claims	<u>None</u>	NO

Industrial Applicability (IA)

Claims	<u>1-40</u>	YES
Claims	<u>None</u>	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-40 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest the coexpression of the nucleic acid sequence encoding protein X and diol or glycerol dehydratase in a microorganism to enhance the stability and the activity of the dehydratase *in vivo*. The transformed microorganism is more efficient in producing 1,3-propanediol.

----- NEW CITATIONS -----
NONE

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US97/20873

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below: IPC(6): C12P 7/18; C12N 1/14, 1/20, 9/96 and US Cl.: 435/158, 188, 252.3, 252.31, 252.33, 252.34, 252.35, 254.11, 254.2, 254.21, 254.22, 254.23, 254.3

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From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT
OR THE DECLARATION

(PCT Rule 44.1)

To:

GENENCOR INTERNATIONAL, INC.
Attn. GLAISTER, D.J.
925 Page Mill Road
Palo Alto, California 94304-1013
UNITED STATES OF AMERICA

Date of mailing
(day/month/year)

08/05/1998

Applicant's or agent's file reference

GC369-2-PCT

FOR FURTHER ACTION

See paragraphs 1 and 4 below

International application No.

PCT/US 97/20873

International filing date
(day/month/year)

13/11/1997

Applicant

GENENCOR INTERNATIONAL INC. et al.

1. ☒ The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.
Filing of amendments and statement under Article 19

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
- ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. Further action(s): The applicant is reminded of the following:

Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority
European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Mireille Claudepierre

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NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 18 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

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NOTES TO FORM PCT/ISA/220 (continued)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume I of the PCT Applicant's Guide.

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference GC369-2-PCT	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/US 97/ 20873	International filing date (day/month/year) 13/11/1997	(Earliest) Priority Date (day/month/year) 13/11/1996
Applicant GENENCOR INTERNATIONAL INC, et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. ☐ Certain claims were found unsearchable (see Box I).

2. ☐ Unity of invention is lacking (see Box II).

3. ☒ The international application contains disclosure of a nucleotide and/or amino acid sequence listing and the international search was carried out on the basis of the sequence listing

☒ filed with the international application.

☐ furnished by the applicant separately from the international application.

☐ but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.

☐ Transcribed by this Authority

4. With regard to the title, ☒ the text is approved as submitted by the applicant

☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

☒ the text is approved as submitted by the applicant

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is:

Figure No. _____ ☐ as suggested by the applicant;

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 97/20873

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/53 C12N15/55 C12N15/60 C12P7/18 C12N9/04
C12N9/16 C12N9/88

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X, L	WO 96 35796 A (DU PONT ; GENENCOR INT (US); LAFFEND LISA ANNE (US); NAGARAJAN VASA) 14 November 1996 see the whole document see abstract see examples 2-5 see examples 22, 23 see page 62, paragraph 2	1-40
P, A	WO 96 35795 A (DU PONT ; NAGARAJAN VASANTHA (US); NAKAMURA CHARLES EDWIN (US)) 14 November 1996 see abstract see page 9 see examples 1-3	1-13, 15-40
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

21 April 1998

Date of mailing of the international search report

08/05/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel: (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Lejeune, R

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>TOBIMATSU T. ET AL.: "Molecular cloning, sequencing, and expression of the genes encoding adenosylcobalamin-dependent diol dehydrase of <i>Klebsiella oxytoca</i>." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 13, 31 March 1995, pages 7142-7148, XP002062740 cited in the application see page 7147, column 2, paragraph 2 see page 7145, column 1, line 1 - page 7146, column 1, line 4 see abstract</p>	1,5,7,8, 10,11
A	<p>TOBIMATSU T. ET AL.: "Cloning, sequencing and high level expression of the genes encoding adenosylcobalamin-dependent glycerol dehydrase of <i>Klebsiella pneumoniae</i>." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 37, 13 September 1996, pages 22352-22357, XP002057923 see abstract see page 22356, column 1, line 2 - line 5 see page 22356, column 2, paragraph 4 - page 22357, column 1, paragraph 1</p>	1,4,6,8, 10,11,15
A	<p>TONG I.T. ET AL.: "1,3-propanediol production by <i>Escherichia coli</i> expressing genes from the <i>Klebsiella pneumoniae</i> dha regulon." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 57, 1991, pages 3541-3546, XP002062741 see abstract</p>	1-4,6,8, 10,11
A	<p>SEYFRIED M. ET AL.: "Cloning, sequencing, and overexpression of the genes encoding coenzyme B12-dependent glycerol dehydratase of <i>Citrobacter freundii</i>." JOURNAL OF BACTERIOLOGY, vol. 178, no. 19, October 1996, pages 5793-5796, XP002062742 cited in the application see page 5794; figure 1 see page 5793, column 2, paragraph 5 see abstract</p>	1-4,6

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PCT/US 97/20873

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9635796 A	14-11-96	US 5686276 A	11-11-97
		AU 5678996 A	29-11-96
		EP 0826057 A	04-03-98
WO 9635795 A	14-11-96	US 5633362 A	27-05-97
		AU 5722996 A	29-11-96
		EP 0827543 A	11-03-98

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/53, 15/55, 15/60, C12P 7/18, C12N 9/04, 9/16, 9/88	A3	(11) International Publication Number: WO 98/21341 (43) International Publication Date: 22 May 1998 (22.05.98)
(21) International Application Number: PCT/US97/20873 (22) International Filing Date: 13 November 1997 (13.11.97) (30) Priority Data: 60/030,601 13 November 1996 (13.11.96) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/030,601 (CIP) Filed on Not furnished (71) Applicant (for all designated States except US): GENENCOR INTERNATIONAL, INC. [US/US]; 4 Cambridge Place, 1870 South Winton Road, Rochester, NY 14618 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): DUNN-COLEMAN, Nigel, S. [GB/US]; 142 Johnson Avenue, Los Gatos, CA 95032 (US). DIAZ-TORRES, Maria [ES/US]; 58 North El Camino Real, San Mateo, CA 94401 (US). CHASE, Matthew, W. [US/US]; 2211-27 Hastings Drive, Belmont, CA 94002 (US). TRIMBUR, Donald [US/US]; 349 Orchard Avenue, Redwood City, CA 94601 (US).	(74) Agent: GLAISTER, Debra, J.; Genencor International, Inc., Page Mill Road, Palo Alto, CA 94304-1013 (US). (81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>With amended claims.</i> (88) Date of publication of the international search report: 25 June 1998 (25.06.98) Date of publication of the amended claims: 30 July 1998 (30.07.98)	
(54) Title: METHOD FOR THE RECOMBINANT PRODUCTION OF 1,3-PROPANEDIOL (57) Abstract <p>The present invention provides an improved method for the production of 1,3-propanediol from a variety of carbon sources is an organism comprising DNA encoding protein X of a dehydratase or protein X in combination with at least one of protein 1, protein 2 and protein 3. The protein X may be isolated from a diol dehydratase or a glycerol dehydratase. The present invention also provides host cells comprising protein X that are capable of increased production of 1,3-propanediol.</p>		

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/53, 15/55, 15/60, C12P 7/18, C12N 9/04, 9/16, 9/88	A3	(11) International Publication Number: WO 98/21341 (43) International Publication Date: 22 May 1998 (22.05.98)
(21) International Application Number: PCT/US97/20873 (22) International Filing Date: 13 November 1997 (13.11.97) (30) Priority Data: 60/030,601 13 November 1996 (13.11.96) US (71) Applicant (for all designated States except US): GENENCOR INTERNATIONAL, INC. [US/US]; 4 Cambridge Place, 1870 South Winton Road, Rochester, NY 14618 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): DUNN-COLEMAN, Nigel, S. [GB/US]; 142 Johnson Avenue, Los Gatos, CA 95032 (US). DIAZ-TORRES, Maria [ES/US]; 58 North El Camino Real, San Mateo, CA 94401 (US). CHASE, Matthew, W. [US/US]; 2211-27 Hastings Drive, Belmont, CA 94002 (US). TRIMBUR, Donald [US/US]; 349 Orchard Avenue, Redwood City, CA 94601 (US). (74) Agent: GLAISTER, Debra, J.; Genencor International, Inc., Page Mill Road, Palo Alto, CA 94304-1013 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 25 June 1998 (25.06.98)
(54) Title: METHOD FOR THE RECOMBINANT PRODUCTION OF 1,3-PROPANEDIOL (57) Abstract The present invention provides an improved method for the production of 1,3-propanediol from a variety of carbon sources is an organism comprising DNA encoding protein X of a dehydratase or protein X in combination with at least one of protein 1, protein 2 and protein 3. The protein X may be isolated from a diol dehydratase or a glycerol dehydratase. The present invention also provides host cells comprising protein X that are capable of increased production of 1,3-propanediol.		

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510 Rec'd PCT/PTO 13 MAY 1999

METHOD FOR THE RECOMBINANT
PRODUCTION OF 1,3-PROPANEDIOL

Related Applications

5 The present application is a continuation-in-part application of United States Provisional Application 60/030,601 filed November 13, 1996, hereby incorporated herein in its entirety.

Field of Invention

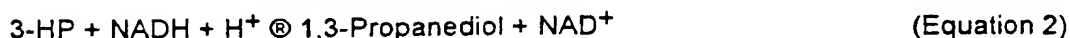
10 The present invention relates to the field of molecular biology and specifically to improved methods for the production of 1,3-propanediol in host cells. In particular, the present invention describes components of gene clusters associated with 1,3-propanediol production in host cells, including protein X, and protein 1, protein 2 and protein 3. More specifically the present invention describes the expression of cloned genes encoding protein X, protein 1, protein 2 and protein 3, either separately or together, for the enhanced production of 1,3-propanediol in host cells.

Background

15 1,3-Propanediol is a monomer having potential utility in the production of polyester fibers and the manufacture of polyurethanes and cyclic compounds.

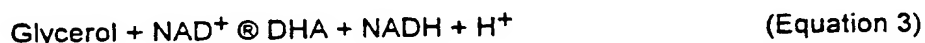
A variety of chemical routes to 1,3-propanediol are known. For example ethylene oxide may be converted to 1,3-propanediol over a catalyst in the presence of phosphine, water, carbon monoxide, hydrogen and an acid, by the catalytic solution phase hydration of acrolein followed by
20 reduction, or from hydrocarbons such as glycerol, reacted in the presence of carbon monoxide and hydrogen over catalysts having atoms from group VIII of the periodic table. Although it is possible to generate 1,3-propanediol by these methods, they are expensive and generate waste streams containing environmental pollutants.

It has been known for over a century that 1,3-propanediol can be produced from the
25 fermentation of glycerol. Bacterial strains able to produce 1,3-propanediol have been found, for example, in the groups *Citrobacter*, *Clostridium*, *Enterobacter*, *Ilyobacter*, *Klebsiella*, *Lactobacillus*, and *Pelobacter*. In each case studied, glycerol is converted to 1,3-propanediol in a two step, enzyme catalyzed reaction sequence. In the first step, a dehydratase catalyzes the conversion of glycerol to 3-hydroxypropionaldehyde (3-HP) and water (Equation 1). In the second
30 step, 3-HP is reduced to 1,3-propanediol by a NAD⁺-linked oxidoreductase (Equation 2).



35 The 1,3-propanediol is not metabolized further and, as a result, accumulates in high concentration in the media. The overall reaction consumes a reducing equivalent in the form of a cofactor, reduced b-nicotinamide adenine dinucleotide (NADH), which is oxidized to nicotinamide adenine dinucleotide (NAD⁺).

The production of 1,3-propanediol from glycerol is generally performed under anaerobic conditions using glycerol as the sole carbon source and in the absence of other exogenous reducing equivalent acceptors. Under these conditions, in for example, strains of *Citrobacter*, *Clostridium*, and *Klebsiella*, a parallel pathway for glycerol operates which first involves oxidation of glycerol to dihydroxyacetone (DHA) by a NAD⁺- (or NADP⁺-) linked glycerol dehydrogenase (Equation 3). The DHA, following phosphorylation to dihydroxyacetone phosphate (DHAP) by a DHA kinase (Equation 4), becomes available for biosynthesis and for supporting ATP generation via, for example, glycolysis.



In contrast to the 1,3-propanediol pathway, this pathway may provide carbon and energy to the cell and produces rather than consumes NADH.

In *Klebsiella pneumoniae* and *Citrobacter freundii*, the genes encoding the functionally linked activities of glycerol dehydratase (*dhaB*), 1,3-propanediol oxidoreductase (*dhaT*), glycerol dehydrogenase (*dhaD*), and dihydroxyacetone kinase (*dhaK*) are encompassed by the *dha* regulon. The *dha* regulons from *Citrobacter* and *Klebsiella* have been expressed in *Escherichia coli* and have been shown to convert glycerol to 1,3-propanediol. Glycerol dehydratase (E.C. 4.2.1.30) and diol [1,2-propanediol] dehydratase (E.C. 4.2.1.28) are related but distinct enzymes that are encoded by distinct genes. In *Salmonella typhimurium* and *Klebsiella pneumoniae*, diol dehydratase is associated with the *pdu* operon, see Bobik et al., 1992, J. Bacteriol. 174:2253-2266 and United States patent 5,633,362. Tobimatsu, et al., 1996, J. Biol. Chem. 271: 22352-22357 disclose the *K. pneumoniae* gene encoding glycerol dehydratase protein X identified as ORF 4; Segfried et al., 1996, J. Bacteriol. 178: 5793-5796 disclose the *C. freundii* glycerol dehydratase gene encoding protein X identified as ORF Z. Tobimatsu et al., 1995, J. Biol. Chem. 270:7142-7148 disclose the diol dehydratase submits α , β and γ and illustrate the presence of orf 4. Luers (1997, FEMS Microbiology Letters 154:337-345) disclose the amino acid sequence of protein 1, protein 2 and protein 3 of *Clostridium pasteurianum*.

Biological processes for the preparation of glycerol are known. The overwhelming majority of glycerol producers are yeasts, but some bacteria, other fungi and algae are also known to produce glycerol. Both bacteria and yeasts produce glycerol by converting glucose or other carbohydrates through the fructose-1,6-bisphosphate pathway in glycolysis or by the Embden Meyerhof Parnas pathway, whereas, certain algae convert dissolved carbon dioxide or bicarbonate in the chloroplasts into the 3-carbon intermediates of the Calvin cycle. In a series of steps, the 3-carbon intermediate, phosphoglyceric acid, is converted to glyceraldehyde 3-phosphate which can be readily interconverted to its keto isomer dihydroxyacetone phosphate and ultimately to glycerol.

Specifically, the bacteria *Bacillus licheniformis* and *Lactobacillus lycopersica* synthesize glycerol, and glycerol production is found in the halotolerant algae *Dunaliella sp.* and *Asteromonas gracilis* for protection against high external salt concentrations (Ben-Amotz et al., *Experientia* 38, 49-52, (1982)). Similarly, various osmotolerant yeasts synthesize glycerol as a protective measure. Most strains of *Saccharomyces* produce some glycerol during alcoholic fermentation, and this can be increased physiologically by the application of osmotic stress (Albertyn et al., *Mol. Cell. Biol.* 14, 4135-4144, (1994)). Earlier this century commercial glycerol production was achieved by the use of *Saccharomyces* cultures to which "steering reagents" were added such as sulfites or alkalis. Through the formation of an inactive complex, the steering agents block or inhibit the conversion of acetaldehyde to ethanol; thus, excess reducing equivalents (NADH) are available to or "steered" towards DHAP for reduction to produce glycerol. This method is limited by the partial inhibition of yeast growth that is due to the sulfites. This limitation can be partially overcome by the use of alkalis which create excess NADH equivalents by a different mechanism. In this practice, the alkalis initiated a Cannizzarro disproportionation to yield ethanol and acetic acid from two equivalents of acetaldehyde.

The gene encoding glycerol-3-phosphate dehydrogenase (DAR1, GPD1) has been cloned and sequenced from *S. diastaticus* (Wang et al., *J. Bact.* 176, 7091-7095, (1994)). The DAR1 gene was cloned into a shuttle vector and used to transform *E. coli* where expression produced active enzyme. Wang et al. (supra) recognize that DAR1 is regulated by the cellular osmotic environment but do not suggest how the gene might be used to enhance 1,3-propanediol production in a recombinant organism.

Other glycerol-3-phosphate dehydrogenase enzymes have been isolated: for example, sn-glycerol-3-phosphate dehydrogenase has been cloned and sequenced from *S. cerevisiae* (Larason et al., *Mol. Microbiol.* 10, 1101, (1993)) and Albertyn et al., (*Mol. Cell. Biol.* 14, 4135, (1994)) teach the cloning of GPD1 encoding a glycerol-3-phosphate dehydrogenase from *S. cerevisiae*. Like Wang et al. (supra), both Albertyn et al. and Larason et al. recognize the osmo-sensitivity of the regulation of this gene but do not suggest how the gene might be used in the production of 1,3-propanediol in a recombinant organism.

As with G3PDH, glycerol-3-phosphatase has been isolated from *Saccharomyces cerevisiae* and the protein identified as being encoded by the GPP1 and GPP2 genes (Norbeck et al., *J. Biol. Chem.* 271, 13875, (1996)). Like the genes encoding G3PDH, it appears that GPP2 is osmosensitive.

Although biological methods of both glycerol and 1,3-propanediol production are known, it has never been demonstrated that the entire process can be accomplished by a single recombinant organism.

Neither the chemical nor biological methods described above for the production of 1,3-propanediol are well suited for industrial scale production since the chemical processes are energy intensive and the biological processes require the expensive starting material, glycerol. A

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method requiring low energy input and an inexpensive starting material is needed. A more desirable process would incorporate a microorganism that would have the ability to convert basic carbon sources such as carbohydrates or sugars to the desired 1,3-propanediol end-product.

Although a single organism conversion of fermentable carbon source other than glycerol or dihydroxyacetone to 1,3-propanediol would be desirable, it has been documented that there are significant difficulties to overcome in such an endeavor. For example, Gottschalk et al. (EP 373 230) teach that the growth of most strains useful for the production of 1,3-propanediol, including *Citrobacter freundii*, *Clostridium autobutylicum*, *Clostridium butylicum*, and *Klebsiella pneumoniae*, is disturbed by the presence of a hydrogen donor such as fructose or glucose. Strains of *Lactobacillus brevis* and *Lactobacillus buchneri*, which produce 1,3-propanediol in co-fermentations of glycerol and fructose or glucose, do not grow when glycerol is provided as the sole carbon source, and, although it has been shown that resting cells can metabolize glucose or fructose, they do not produce 1,3-propanediol. (Veiga DA Cunha et al., *J. Bacteriol.* 174, 1013 (1992)). Similarly, it has been shown that a strain of *Ilyobacter polytropus*, which produces 1,3-propanediol when glycerol and acetate are provided, will not produce 1,3-propanediol from carbon substrates other than glycerol, including fructose and glucose. (Steib et al., *Arch. Microbiol.* 140, 139 (1984)). Finally Tong et al. (*Appl. Biochem. Biotech.* 34, 149 (1992)) has taught that recombinant *Escherichia coli* transformed with the *dha* regulon encoding glycerol dehydratase does not produce 1,3-propanediol from either glucose or xylose in the absence of exogenous glycerol.

Attempts to improve the yield of 1,3-propanediol from glycerol have been reported where co-substrates capable of providing reducing equivalents, typically fermentable sugars, are included in the process. Improvements in yield have been claimed for resting cells of *Citrobacter freundii* and *Klebsiella pneumoniae* DSM 4270 cofermenting glycerol and glucose (Gottschalk et al., *supra.*, and Tran-Dinh et al., DE 3734 764); but not for growing cells of *Klebsiella pneumoniae* ATCC 25955 cofermenting glycerol and glucose, which produced no 1,3-propanediol (I-T. Tong, Ph.D. Thesis, University of Wisconsin-Madison (1992)). Increased yields have been reported for the cofermentation of glycerol and glucose or fructose by a recombinant *Escherichia coli*; however, no 1,3-propanediol is produced in the absence of glycerol (Tong et al., *supra.*). In these systems, single organisms use the carbohydrate as a source of generating NADH while providing energy and carbon for cell maintenance or growth. These disclosures suggest that sugars do not enter the carbon stream that produces 1,3-propanediol. In no case is 1,3-propanediol produced in the absence of an exogenous source of glycerol. Thus the weight of literature clearly suggests that the production of 1,3-propanediol from a carbohydrate source by a single organism is not possible.

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The weight of literature regarding the role of protein X in 1,3-propanediol production by a host cell is at best confusing. Prior to the availability of gene information, McGee et al., 1982, Biochem. Biophys. Res. Comm. 108: 547-551, reported diol dehydratase from *K. pneumoniae* ATCC 8724 to be composed of four subunits identified by size (60K, 51K, 29K, and 15K daltons) and N-terminal amino acid sequence. In direct contrast to McGee, Tobimatsu et al. 1995, *supra*, report the cloning, sequencing and expression of diol dehydratase from the same organism and find no evidence linking the 51K dalton polypeptide to dehydratase. Tobimatsu et al. 1996, *supra*, conclude that the protein X polypeptide is not a subunit of glycerol dehydratase, in contrast to GenBank Accession Number U30903 where protein X is described as a large subunit of glycerol dehydratase. Seyfried et al., *supra*, report that a deletion of 192 bp from the 3' end of *orfZ* (protein X) had no effect on enzyme activity and conclude that *orfZ* does not encode a subunit required for dehydratase activity. Finally, Skraly, F.A. (1997, Thesis entitled "Metabolic Engineering of an Improved 1,3-Propanediol Fermentation") disclose a loss of glycerol dehydratase activity in one experiment where recombinant ORF3 (protein X) was disrupted creating a large fusion protein but not in another experiment where 1,3-propanediol production from glycerol was diminished compared to a control where ORF3 was intact.

The problem to be solved by the present invention is the biological production of 1,3-propanediol by a single recombinant organism from an inexpensive carbon substrate such as glucose or other sugars in commercially feasible quantities. The biological production of 1,3-propanediol requires glycerol as a substrate for a two step sequential reaction in which a dehydratase enzyme (typically a coenzyme B₁₂-dependent dehydratase) converts glycerol to an intermediate, 3-hydroxypropionaldehyde, which is then reduced to 1,3-propanediol by a NADH- (or NADPH) dependent oxidoreductase. The complexity of the cofactor requirements necessitates the use of a whole cell catalyst for an industrial process which utilizes this reaction sequence for the production of 1,3-propanediol. Furthermore, in order to make the process economically viable, a less expensive feedstock than glycerol or dihydroxyacetone is needed and high production levels are desirable. Glucose and other carbohydrates are suitable substrates, but, as discussed above, are known to interfere with 1,3-propanediol production. As a result no single organism has been shown to convert glucose to 1,3-propanediol.

Applicants have solved the stated problem and the present invention provides for bioconverting a fermentable carbon source directly to 1,3-propanediol using a single recombinant organism. Glucose is used as a model substrate and the bioconversion is applicable to any existing microorganism. Microorganisms harboring the genes encoding protein X and protein 1, protein 2 and protein 3 in addition to other proteins associated with the production of 1,3-propanediol, are able to convert glucose and other sugars through the glycerol degradation pathway to 1,3-propanediol with good yields and selectivities. Furthermore, the present invention

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may be generally applied to include any carbon substrate that is readily converted to 1) glycerol, 2) dihydroxyacetone, or 3) C₃ compounds at the oxidation state of glycerol (e.g., glycerol 3-phosphate) or 4) C₃ compounds at the oxidation state of dihydroxyacetone (e.g., dihydroxyacetone phosphate or glyceraldehyde 3-phosphate).

5 **Summary of the Invention**

The present invention relates to improved methods for the production of 1,3-propanediol from a single microorganism. The present invention is based, in part, upon the unexpected discovery that the presence of a gene encoding protein X in a microorganism containing at least one gene encoding a dehydratase activity and capable of producing 1,3-propanediol is associated with the *in vivo* reactivation of dehydratase activity and increased production of 1,3-propanediol in the microorganism. The present invention is also based, in part, upon the unexpected discovery that the presence of a gene encoding protein X and at least one gene encoding a protein selected from the group consisting of protein 1, protein 2 and protein 3 in host cells containing at least one gene encoding a dehydratase activity and capable of producing 1,3-propanediol is associated with *in vivo* reactivation of the dehydratase activity and increased yields of 1,3-propanediol in the microorganism.

Accordingly, the present invention provides an improved method for the production of 1,3-propanediol from a microorganism capable of producing 1,3-propanediol, said microorganism comprising at least one gene encoding a dehydratase activity, the method comprising the steps of introducing a gene encoding protein X into the organism to create a transformed organism; and culturing the transformed organism in the presence of at least one carbon source capable of being converted to 1,3 propanediol in said transformed host organism and under conditions suitable for the production of 1,3 propanediol wherein the carbon source is selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and a one carbon substrate.

In a preferred embodiment, the method for improved production of 1,3-propanediol further comprises introducing at least one gene encoding a protein selected from the group consisting of protein 1, protein 2 and protein 3 into the organism. The microorganism may further comprise at least one of (a) a gene encoding a glycerol-3-phosphate dehydrogenase activity; (b) a gene encoding a glycerol-3-phosphatase activity; and (c) a gene encoding 1,3-propanediol oxidoreductase activity into the microorganism. Gene(s) encoding a dehydratase activity, protein X, proteins 1, 2 or 3 or other genes necessary for the production of 1,3-propanediol may be stably maintained in the host cell genome or may be on replicating plasmids residing in the host microorganism.

The method optionally comprises the step of recovering the 1,3 propanediol. In one aspect of the present invention, the carbon source is glucose.

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The microorganism is selected from the group of genera consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*,
5 *Streptomyces* and *Pseudomonas*.

In one aspect, protein X is derived from a glyceol dehydratase gene cluster and in another aspect, protein X is derived from a diol dehydratase gene cluster. The gene encoding the dehydratase activity may be homologous to the microorganism or heterologous to the microorganism. In one embodiment, the glycerol dehydratase gene cluster is derived from an
10 organism selected from the genera consisting of *Klebsiella* and *Citrobacter*. In another embodiment, the diol dehydratase gene cluster is derived from an organism selected from the genera consisting of *Klebsiella*, *Clostridium* and *Salmonella*.

In another aspect, the present invention provides a recombinant microorganism comprising at least one gene encoding a dehydratase activity; at least one gene encoding a
15 glycerol-3-phosphatase; and at least one gene encoding protein X, wherein said microorganism is capable of producing 1,3-propanediol from a carbon source. The carbon source may be selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and a one carbon substrate. In a further embodiment, the microorganism further comprises a gene encoding a cytosolic glycerol-3-phosphate dehydrogenase. In another embodiment, the
20 recombinant microorganism further comprises at least one gene encoding a protein selected from the group consisting of protein 1, protein 2 and protein 3. The microorganism is selected from the group consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*,
25 *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*. In one aspect, protein X is derived from a glycerol dehydratase gene cluster. In another aspect, protein X is derived from a diol dehydratase gene cluster. In one aspect, the dehydratase activity is heterologous to said microorganism and in another aspect, the dehydratase activity is homologous to said microorganism.

The present invention also provides a method for the *in vivo* reactivation of a dehydratase
30 activity in a microorganism capable of producing 1,3-propanediol and containing at least one gene encoding a dehydratase activity, comprising the step of introducing a gene encoding protein X into said microorganism. The microorganism is selected from the group consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*,
35 *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*,

Hansenula, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*.

In one aspect, the gene encoding the dehydratase activity is heterologous to said microorganism and in another aspect, the gene encoding the dehydratase activity is homologous to said microorganism. In one embodiment, the gene encoding protein X is derived from a glycerol dehydratase gene cluster and in another embodiment, the gene encoding protein X is derived from a diol dehydratase gene cluster.

The present invention also provides expression vectors and host cells containing genes encoding protein X, protein 1, protein 2 and protein 3.

One advantage of the method of production of 1,3-propanediol according to the present invention is the unexpected increased production of 1,3-propanediol in a host cell capable of producing 1,3-propanediol in the presence of nucleic acid encoding protein X as compared to the host cell lacking nucleic acid encoding protein X. As demonstrated *infra*, a host cell containing nucleic acid encoding dhaB 1, 2 and 3 and protein X is able to produce significantly more 1,3-propanediol than a host cell containing nucleic acid encoding dhaB 1, 2 and 3 and lacking X.

Another advantage of the present invention as demonstrated *infra*, is that the presence of nucleic acid encoding protein X along with nucleic acid encoding at least one of protein 1, protein 2 and protein 3 in a host cell capable of producing 1,3-propanediol gives the unexpected result of increased production of 1,3-propanediol in the host cell over 1,3-propanediol production in the host cell lacking nucleic acid encoding protein X along with nucleic acid encoding at least one of protein 1, protein 2 and protein 3.

Yet another advantage of the method of production of the present invention as shown *infra* is the *in vivo* reactivation of the dehydratase activity in a microorganism that is associated with the presence of nucleic acid encoding protein X in the microorganism.

Brief Description of the Drawings

Figure 1 illustrates components of the glycerol dehydratase gene cluster from *Klebsiella pneumoniae* on plasmid pHK28-26 (SEQ ID NO:19). In this figure, orfY encodes protein 1, orfX encodes protein 2 and orfW encodes protein 3. DhaB-X refers to protein X.

Figures 2A-2G illustrates the nucleotide and amino acid sequence of *Klebsiella pneumoniae* glycerol dehydratase protein X (dhab4) (SEQ ID NO:59).

Figure 3 illustrates the amino acid alignment of *Klebsiella pneumoniae* protein 1 (SEQ ID NO: 61) and *Citrobacter freundii* protein1 (SEQ ID NO: 60) (designated in Figure 3 as orfY).

Figure 4 illustrates the amino acid alignment of *Klebsiella pneumoniae* protein 2 (SEQ ID NO: 63) and *Citrobacter freundii* protein 2 (SEQ ID NO: 62) (designated in Figure 4 as orfX).

Figure 5 illustrates the amino acid alignment of *Klebsiella pneumoniae* protein 3 (SEQ ID NO: 64) and *Citrobacter freundii* protein 3 (SEQ ID NO: 65) (designated in Figure 5 as orfW).

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Figure 6 illustrates the in situ reactivation comparison of plasmids pHK28-26 (which contains dhaB subunits 1, 2 and 3 as well as protein X and the open reading frames encoding protein 1, protein 2 and protein 3) vs. pDT24 (which contains dhaB subunits 1, 2 and 3 as well as protein X) in *E.coli* DH5 α cells.

Figure 7 illustrate the in situ reactivation comparison of plasmids pM7 (containing genes encoding dhaB subunits 1, 2 and 3 and protein X) vs. Plasmid pM11 (containing genes encoding dhaB subunits 1, 2 and 3) in *E.coli* DH5 α cells.

Figures 8A-8E illustrates the nucleic acid (SEQ ID NO: 66) and amino acid (SEQ ID NO: 67) sequence of *K. pneumoniae* diol dehydratase gene cluster protein X.

Figure 9 illustrates a standard 10 liter fermentation for 1,3 propandiol production using *E. coli* FM5/pDT24 (FM5 described in Amgen patent US 5,494,816 , ATCC accession No. 53911).

Figure 10 illustrates a standard 10 liter fermentation for 1,3 propandiol production using *E. coli* DH5 α /pHK28-26.

Brief Description of Biological Deposits and Sequence Listing

The transformed *E. coli* W2042 (comprising the *E. coli* host W1485 and plasmids pDT20 and pAH42) containing the genes encoding glycerol-3-phosphate dehydrogenase (G3PDH) and glycerol-3-phosphatase (G3P phosphatase), glycerol dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase (*dhaT*) was deposited on 26 September 1996 with the ATCC under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purpose of Patent Procedure and is designated as ATCC 98188.

S. cerevisiae YPH500 harboring plasmids pMCK10, pMCK17, pMCK30 and pMCK35 containing genes encoding glycerol-3-phosphate dehydrogenase (G3PDH) and glycerol-3-phosphatase (G3P phosphatase), glycerol dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase (*dhaT*) was deposited on 26 September 1996 with the ATCC under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purpose of Patent Procedure and is designated as ATCC 74392.

E.coli DH5 α containing pKP1 which has about 35kb of a *Klebsiella* genome which contains the glycerol dehydratase, protein X and proteins 1, 2 and 3 was deposited on 18 April 1995 with the ATCC under the terms of the Budapest Treaty and was designated ATCC 69789. *E.coli* DH5 α containing pKP4 containing a portion of the *Klebsiella* genome encoding diol dehydratase enzyme, including protein X was deposited on 18 April 1995 with the ATCC under the terms of the Budapest Treaty and was designated ATCC 69790.

"ATCC" refers to the American Type Culture Collection international depository located at 12301 Parklawn Drive, Rockville, MD 20852 U.S.A. The designations refer to the accession number of the deposited material.

Detailed Description of the Invention

The present invention relates to the production of 1,3-propanediol in a single microorganism and provides improved methods for production of 1,3-propanediol from a fermentable carbon source in a single recombinant organism. The method incorporates a microorganism capable of producing 1,3-propanediol comprising either homologous or heterologous genes encoding dehydratase (*dhaB*), at least one gene encoding protein X and optionally at least one of the genes encoding a protein selected from the group consisting of protein 1, protein 2 and protein 3. Optionally, the microorganism contains at least one gene encoding glycerol-3-phosphate dehydrogenase, glycerol-3-phosphatase and 1,3-propanediol oxidoreductase (*dhaT*). The recombinant microorganism is contacted with a carbon substrate and 1,3-propanediol is isolated from the growth media.

The present method provides a rapid, inexpensive and environmentally responsible source of 1,3-propanediol monomer useful in the production of polyesters and other polymers.

The following definitions are to be used to interpret the claims and specification.

The term "dehydratase gene cluster" or "gene cluster" refers to the set of genes which are associated with 1,3-propanediol production in a host cell and is intended to encompass glycerol dehydratase gene clusters as well as diol dehydratase gene clusters. The *dha* regulon refers to a glycerol dehydratase gene cluster, as illustrated in Figure 1 which includes regulatory regions.

The term "regenerating the dehydratase activity" or "reactivating the dehydratase activity" refers to the phenomenon of converting a dehydratase not capable of catalysis of a substrate to one capable of catalysis of a substrate or to the phenomenon of inhibiting the inactivation of a dehydratase or the phenomenon of extending the useful half-life of the dehydratase enzyme *in vivo*.

The terms "glycerol dehydratase" or "dehydratase enzyme" or "dehydratase activity" refer to the polypeptide(s) responsible for an enzyme activity that is capable of isomerizing or converting a glycerol molecule to the product 3-hydroxypropionaldehyde. For the purposes of the present invention the dehydratase enzymes include a glycerol dehydratase (GenBank U09771, U30903) and a diol dehydratase (GenBank D45071) having preferred substrates of glycerol and 1,2-propanediol, respectively. Glycerol dehydratase of *K. pneumoniae* ATCC 25955 is encoded by the genes *dhaB1*, *dhaB2*, and *dhaB3* identified as SEQ ID NOS:1, 2 and 3, respectively. The *dhaB1*, *dhaB2*, and *dhaB3* genes code for the a, b, and c subunits of the glycerol dehydratase enzyme, respectively.

The phrase "protein X of a dehydratase gene cluster" or "dhaB protein X" or "protein X" refers to a protein that is comparable to protein X of the *Klebsiella pneumoniae* dehydratase gene cluster as shown in Figure 2 or alternatively comparable to protein X of *Klebsiella pneumoniae* diol dehydratase gene cluster as shown in Figure 8. Preferably protein X is capable of increasing

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the production of 1,3-propanediol in a host organism over the production of 1,3-propanediol in the absence of protein X in the host organism. Being comparable means that DNA encoding the protein is either in the same structural location as DNA encoding *Klebsiella* protein X with respect to *Klebsiella* dhaB1, dhaB2 and dhaB3, i.e., DNA encoding protein X is 3' to nucleic acid encoding dhaB1-B3, or that protein X has overall amino acid similarity to either *Klebsiella* diol or glycerol dehydratase protein X. The present invention encompasses protein X molecules having at least 50%; or at least 65 %; or at least 80%; or at least 90% or at least 95% similarity to the protein X of *K. pneumoniae* glycerol or diol dehydratase or the *C. freundii* protein X.

Included within the term "protein X" is protein X, also referred to as ORF Z, from *Citrobacter* dha regulon (Segfried M. 1996, J. Bacteriol. 178: 5793:5796). The present invention also encompasses amino acid variations of protein X from any microorganism as long as the protein X variant retains its essential functional characteristics of increasing the production of 1,3-propanediol in a host organism over the production of 1,3-propanediol in the host organism in the absence of protein X.

A portion of the *Klebsiella* genome encoding the glycerol dehydratase enzyme activity as well as protein X was transformed into *E.coli* and the transformed *E.coli* was deposited on 18 April 1995 with the ATCC under the terms of the Budapest Treaty and was designated as ATCC accession number 69789. A portion of the *Klebsiella* genome encoding the diol dehydratase enzyme activity as well as protein X was transformed into *E.coli* and the transformed *E.coli* was deposited on 18 April 1995 with the ATCC under the terms of the Budapest Treaty and was designated as ATCC accession number 69790.

Klebsiella glycerol dehydratase protein X is found at bases 9749-11572 of SEQ ID NO:19, counting the first base of dhaK as position number 1. *Citrobacter freundii* (ATCC accession number CFU09771) nucleic acid encoding protein X is found between positions 11261 and 13072.

The present invention encompasses genes encoding dehydratase protein X that are recombinantly introduced and replicate on a plasmid in the host organism as well as genes that are stably maintained in the host genome. The present invention encompasses a method for enhanced production of 1,3-propanediol wherein the gene encoding protein X is transformed in a host cell together with genes encoding the dehydratase activity and/or other genes necessary for the production of 1,3-propanediol. The gene encoding protein X, dehydratase activity and/or other genes may be on the same or different expression cassettes. Alternatively, the gene encoding protein X may be transformed separately, either before or after genes encoding the dehydratase activity and/or other activities. The present invention encompasses host cell having endogenous nucleic acid encoding protein X as well as host cell lacking endogenous nucleic acid encoding protein X.

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The terms "protein 1", "protein 2" and "protein 3" refer to the proteins encoded in a microorganism that are comparable to protein 1 (SEQ ID NO: 60 or SEQ ID NO: 61)(also referred to as orfY), protein 2 (SEQ ID NO: 62 or SEQ ID NO: 63) (also referred to as orfX) and protein 3 (SEQ ID NO: 64 or SEQ ID NO: 65) (also referred to as orfW), respectively.

5 Preferably, in the presence of protein X, at least one of proteins 1, 2 and 3 is capable of increasing the production of 1,3-propanediol in a host organism over the production of 1,3-propanediol in the absence of protein X and at least one of proteins 1, 2 and 3 in the host organism. Being comparable means that DNA encoding the protein is either in the same structural location as DNA encoding the respective proteins, as shown in Figure 1, or that the
10 respective proteins have overall amino acid similarity to the respective SEQ ID NOS shown in Figures 3, 4 and 5.

The present invention encompasses protein 1 molecules having at least 50%; or at least 65 %; or at least 80%; or at least 90% or at least 95% similarity to SEQ ID NO: 60 or SEQ ID NO: 61. The present invention encompasses protein 2 molecules having at least 50%; or at least 65
15 %; or at least 80%; or at least 90% or at least 95% similarity to SEQ ID NO: 62 or SEQ ID NO: 63. The present invention encompasses protein 3 molecules having at least 50%; or at least 65 %; or at least 80%; or at least 90% or at least 95% similarity to SEQ ID NO: 64 or SEQ ID NO: 65.

Included within the terms "protein 1", "protein 2" and "protein 3", respectively, are orfY, orfX and orfW from *Clostridium pasteurianum* (Luers, et al., *supra*) as well as molecules having at
20 least 50%; or at least 65 %; or at least 80%; or at least 90% or at least 95% similarity to *C. pasteurianum* orfY, orfX or orfW. The present invention also encompasses amino acid variations of proteins 1, 2 and 3 from any microorganism as long as the protein variant, in combination with protein X, retains its essential functional characteristics of increasing the production of 1,3-propanediol in a host organism over the production of 1,3-propanediol in the host organism in
25 their absence.

The present invention encompasses a method for enhanced production of 1,3-propanediol wherein the gene(s) encoding at least one of protein 1, protein 2 and protein 3 is transformed in a host cell together with genes encoding protein X, the dehydratase activity and/or other genes necessary for the production of 1,3-propanediol. The gene(s) encoding at least one of proteins 1, 2
30 and 3, protein X, dehydratase activity and/or other genes may be on the same or different expression cassettes. Alternatively, the gene(s) encoding at least one of proteins 1, 2 and 3 may be transformed separately, either before or after genes encoding the dehydratase activity and/or other activities. The present invention encompasses host cell having endogenous nucleic acid encoding protein 1, protein 2 or protein 3 as well as host cell lacking endogenous nucleic acid
35 encoding the proteins.

The terms "oxidoreductase" or "1,3-propanediol oxidoreductase" refer to the polypeptide(s) responsible for an enzyme activity that is capable of catalyzing the reduction of 3-hydroxypropionaldehyde to 1,3-propanediol. 1,3-Propanediol oxidoreductase includes, for example, the polypeptide encoded by the *dhaT* gene (GenBank U09771, U30903) and is identified as SEQ ID NO:4.

The terms "glycerol-3-phosphate dehydrogenase" or "G3PDH" refer to the polypeptide(s) responsible for an enzyme activity capable of catalyzing the conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P). *In vivo* G3PDH may be NADH-, NADPH-, or FAD-dependent. Examples of this enzyme activity include the following: NADH-dependent enzymes (EC 1.1.1.8) are encoded by several genes including GPD1 (GenBank Z74071x2) or GPD2 (GenBank Z35169x1) or GPD3 (GenBank G984182) or DAR1 (GenBank Z74071x2); a NADPH-dependent enzyme (EC 1.1.1.94) is encoded by *gpsA* (GenBank U32164, G466746 (cds 197911-196892), and L45246); and FAD-dependent enzymes (EC 1.1.99.5) are encoded by GUT2 (GenBank Z47047x23) or glpD (GenBank G147838) or glpABC (GenBank M20938).

The terms "glycerol-3-phosphatase" or "sn-glycerol-3-phosphatase" or "d,l-glycerol phosphatase" or "G3P phosphatase" refer to the polypeptide(s) responsible for an enzyme activity that is capable of catalyzing the conversion of glycerol-3-phosphate to glycerol. G3P phosphatase includes, for example, the polypeptides encoded by GPP1 (GenBank Z47047x125) or GPP2 (GenBank U18813x11).

The term "glycerol kinase" refers to the polypeptide(s) responsible for an enzyme activity capable of catalyzing the conversion of glycerol to glycerol-3-phosphate or glycerol-3-phosphate to glycerol, depending on reaction conditions. Glycerol kinase includes, for example, the polypeptide encoded by GUT1 (GenBank U11583x19).

The terms "GPD1", "DAR1", "OSG1", "D2830", and "YDL022W" will be used interchangeably and refer to a gene that encodes a cytosolic glycerol-3-phosphate dehydrogenase and characterized by the base sequence given as SEQ ID NO:5.

The term "GPD2" refers to a gene that encodes a cytosolic glycerol-3-phosphate dehydrogenase and characterized by the base sequence given as SEQ ID NO:6.

The terms "GUT2" and "YIL155C" are used interchangeably and refer to a gene that encodes a mitochondrial glycerol-3-phosphate dehydrogenase and characterized by the base sequence given in SEQ ID NO:7.

The terms "GPP1", "RHR2" and "YIL053W" are used interchangeably and refer to a gene that encodes a cytosolic glycerol-3-phosphatase and characterized by the base sequence given as SEQ ID NO:8.

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The terms "GPP2", "HOR2" and "YER062C" are used interchangeably and refer to a gene that encodes a cytosolic glycerol-3-phosphatase and characterized by the base sequence given as SEQ ID NO:9.

The term "GUT1" refers to a gene that encodes a cytosolic glycerol kinase and characterized by the base sequence given as SEQ ID NO:10.

The terms "function" or "enzyme function" refer to the catalytic activity of an enzyme in altering the energy required to perform a specific chemical reaction. It is understood that such an activity may apply to a reaction in equilibrium where the production of either product or substrate may be accomplished under suitable conditions.

The terms "polypeptide" and "protein" are used interchangeably.

The terms "carbon substrate" and "carbon source" refer to a carbon source capable of being metabolized by host organisms of the present invention and particularly carbon sources selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and one-carbon substrates or mixtures thereof.

The terms "host cell" or "host organism" refer to a microorganism capable of receiving foreign or heterologous genes and of expressing those genes to produce an active gene product.

The terms "foreign gene", "foreign DNA", "heterologous gene" and "heterologous DNA" refer to genetic material native to one organism that has been placed within a host organism by various means. The gene of interest may be a naturally occurring gene, a mutated gene or a synthetic gene.

The terms "recombinant organism" and "transformed host" refer to any organism having been transformed with heterologous or foreign genes or extra copies of homologous genes. The recombinant organisms of the present invention express foreign genes encoding glycerol-3-phosphate dehydrogenase (G3PDH) and glycerol-3-phosphatase (G3P phosphatase), glycerol dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase (*dhaT*) for the production of 1,3-propanediol from suitable carbon substrates.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding) and following (3' non-coding) the coding region. The terms "native" and "wild-type" refer to a gene as found in nature with its own regulatory sequences.

The terms "encoding" and "coding" refer to the process by which a gene, through the mechanisms of transcription and translation, produces an amino acid sequence. It is understood that the process of encoding a specific amino acid sequence includes DNA sequences that may involve base changes that do not cause a change in the encoded amino acid, or which involve base changes which may alter one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. It is therefore understood that the

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invention encompasses more than the specific exemplary sequences. Modifications to the sequence, such as deletions, insertions, or substitutions in the sequence which produce silent changes that do not substantially affect the functional properties of the resulting protein molecule are also contemplated. For example, alteration in the gene sequence which reflect the degeneracy of the genetic code, or which result in the production of a chemically equivalent amino acid at a given site, are contemplated. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. In some cases, it may in fact be desirable to make mutants of the sequence in order to study the effect of alteration on the biological activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity in the encoded products. Moreover, the skilled artisan recognizes that sequences encompassed by this invention are also defined by their ability to hybridize, under stringent conditions (0.1X SSC, 0.1% SDS, 65 °C), with the sequences exemplified herein.

The term "expression" refers to the transcription and translation to gene product from a gene coding for the sequence of the gene product.

The terms "plasmid", "vector", and "cassette" refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitate transformation of a particular host cell. "Expression cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

The terms "transformation" and "transfection" refer to the acquisition of new genes in a cell after the incorporation of nucleic acid. The acquired genes may be integrated into

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chromosomal DNA or introduced as extrachromosomal replicating sequences. The term "transformant" refers to the product of a transformation.

The term "genetically altered" refers to the process of changing hereditary material by transformation or mutation.

5 The term "isolated " refers to a protein or DNA sequence that is removed from at least one component with which it is naturally associated.

The term "homologous" refers to a protein or polypeptide native or naturally occurring in a gram-positive host cell. The invention includes microorganisms producing the homologous protein via recombinant DNA technology.

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CONSTRUCTION OF RECOMBINANT ORGANISMS

Recombinant organisms containing the necessary genes that will encode the enzymatic pathway for the conversion of a carbon substrate to 1,3-propanediol may be constructed using techniques well known in the art. As discussed in Example 9, genes encoding *Klebsiella* dhaB1, dhaB2, dhaB3 and protein X were used to transform *E. coli* DH5a and in Example 10, genes encoding at least one of *Klebsiella* proteins 1, 2 and 3 as well as at least one gene encoding protein X was used to transform *E. coli*.

15 Genes encoding glycerol-3-phosphate dehydrogenase (G3PDH), glycerol-3-phosphatase (G3P phosphatase), glycerol dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase (*dhaT*) were isolated from a native host such as *Klebsiella* or *Saccharomyces* and used to transform host strains such as *E. coli* DH5a, ECL707, AA200, or W1485; the *Saccharomyces cerevisiae* strain YPH500; or the *Klebsiella pneumoniae* strains ATCC 25955 or ECL 2106.

Isolation of Genes

Methods of obtaining desired genes from a bacterial genome are common and well known in the art of molecular biology. For example, if the sequence of the gene is known, suitable genomic libraries may be created by restriction endonuclease digestion and may be screened with probes complementary to the desired gene sequence. Once the sequence is isolated, the DNA may be amplified using standard primer directed amplification methods such as polymerase chain reaction (PCR) (U.S. 4,683,202) to obtain amounts of DNA suitable for transformation using appropriate vectors.

30 Alternatively, cosmid libraries may be created where large segments of genomic DNA (35-45kb) may be packaged into vectors and used to transform appropriate hosts. Cosmid vectors are unique in being able to accommodate large quantities of DNA. Generally, cosmid vectors have at least one copy of the *cos* DNA sequence which is needed for packaging and subsequent circularization of the foreign DNA. In addition to the *cos* sequence these vectors will also contain an origin of replication such as ColE1 and drug resistance markers such as a gene resistant to ampicillin or neomycin. Methods of using cosmid vectors for the transformation of

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suitable bacterial hosts are well described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).

Typically to clone cosmids, foreign DNA is isolated and ligated, using the appropriate restriction endonucleases, adjacent to the *cos* region of the cosmid vector. Cosmid vectors containing the linearized foreign DNA is then reacted with a DNA packaging vehicle such as bacteriophage λ . During the packaging process the *cos* sites are cleaved and the foreign DNA is packaged into the head portion of the bacterial viral particle. These particles are then used to transfect suitable host cells such as *E. coli*. Once injected into the cell, the foreign DNA circularizes under the influence of the *cos* sticky ends. In this manner large segments of foreign DNA can be introduced and expressed in recombinant host cells.

Isolation and cloning of genes encoding glycerol dehydratase (*dhaB*) and 1,3-propanediol oxidoreductase (*dhaT*)

Cosmid vectors and cosmid transformation methods were used within the context of the present invention to clone large segments of genomic DNA from bacterial genera known to possess genes capable of processing glycerol to 1,3-propanediol. Specifically, genomic DNA from *K. pneumoniae* ATCC 25955 was isolated by methods well known in the art and digested with the restriction enzyme *Sau3A* for insertion into a cosmid vector Supercos 1 and packaged using GigapackII packaging extracts. Following construction of the vector *E. coli* XL1-Blue MR cells were transformed with the cosmid DNA. Transformants were screened for the ability to convert glycerol to 1,3-propanediol by growing the cells in the presence of glycerol and analyzing the media for 1,3-propanediol formation.

Two of the 1,3-propanediol positive transformants were analyzed and the cosmids were named pKP1 and pKP2. DNA sequencing revealed extensive homology to the glycerol dehydratase gene (*dhaB*) from *C. freundii*, demonstrating that these transformants contained DNA encoding the glycerol dehydratase gene. Other 1,3-propanediol positive transformants were analyzed and the cosmids were named pKP4 and pKP5. DNA sequencing revealed that these cosmids carried DNA encoding a diol dehydratase gene.

Isolation of genes encoding protein X, protein 1, protein 2 and protein 3

Although the instant invention utilizes the isolated genes from within a *Klebsiella* cosmid, alternate sources of dehydratase genes and protein X and protein 1, protein 2 and protein 3 include, but are not limited to, *Citrobacter*, *Clostridia*, and *Salmonella*. Tobimatsu, et al., 1996, J. Biol. Chem. 271: 22352-22357 disclose the *K. pneumoniae* glycerol dehydratase operon where protein X is identified as ORF 4; Segfried et al., 1996, J. Bacteriol. 178: 5793-5796 disclose the *C. freundii* glycerol dehydratase operon where protein X is identified as ORF Z. Figure 8 discloses

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Klebsiella diol dehydratase protein X and Figures 3, 4 and 5 disclose amino acid sequences of proteins 1, 2 and 3 from *Klebsiella* and *Citrobacter*.

Genes encoding G3PDH and G3P phosphatase

The present invention provides genes suitable for the expression of G3PDH and G3P phosphatase activities in a host cell.

Genes encoding G3PDH are known. For example, GPD1 has been isolated from *Saccharomyces* and has the base sequence given by SEQ ID NO:5, encoding the amino acid sequence given in SEQ ID NO:11 (Wang et al., *supra*). Similarly, G3PDH activity has also been isolated from *Saccharomyces* encoded by GPD2 having the base sequence given in SEQ ID NO:6, encoding the amino acid sequence given in SEQ ID NO:12 (Eriksson et al., *Mol. Microbiol.* 17, 95, (1995).

It is contemplated that any gene encoding a polypeptide responsible for G3PDH activity is suitable for the purposes of the present invention wherein that activity is capable of catalyzing the conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P). Further, it is contemplated that any gene encoding the amino acid sequence of G3PDH as given by any one of SEQ ID NOS:11, 12, 13, 14, 15 and 16 corresponding to the genes GPD1, GPD2, GUT2, *gpsA*, *glpD*, and the α subunit of *glpABC*, respectively, will be functional in the present invention wherein that amino acid sequence encompasses amino acid substitutions, deletions or additions that do not alter the function of the enzyme. It will be appreciated by the skilled person that genes encoding G3PDH isolated from other sources are also suitable for use in the present invention. For example, genes isolated from prokaryotes include GenBank accessions M34393, M20938, L06231, U12567, L45246, L45323, L45324, L45325, U32164, and U39682; genes isolated from fungi include GenBank accessions U30625, U30876 and X56162; genes isolated from insects include GenBank accessions X61223 and X14179; and genes isolated from mammalian sources include GenBank accessions U12424, M25558 and X78593.

Genes encoding G3P phosphatase are known. For example, GPP2 has been isolated from *Saccharomyces cerevisiae* and has the base sequence given by SEQ ID NO:9 which encodes the amino acid sequence given in SEQ ID NO:17 (Norbeck et al., *J. Biol. Chem.* 271, p. 13875, 1996).

It is contemplated that any gene encoding a G3P phosphatase activity is suitable for the purposes of the present invention wherein that activity is capable of catalyzing the conversion of glycerol-3-phosphate to glycerol. Further, it is contemplated that any gene encoding the amino acid sequence of G3P phosphatase as given by SEQ ID NOS:33 and 17 will be functional in the present invention wherein that amino acid sequence encompasses amino acid substitutions, deletions or additions that do not alter the function of the enzyme. It will be appreciated by the skilled person that genes encoding G3P phosphatase isolated from other sources are also

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suitable for use in the present invention. For example, the dephosphorylation of glycerol-3-phosphate to yield glycerol may be achieved with one or more of the following general or specific phosphatases: alkaline phosphatase (EC 3.1.3.1) [GenBank M19159, M29663, U02550 or M33965]; acid phosphatase (EC 3.1.3.2) [GenBank U51210, U19789, U28658 or L20566]; glycerol-3-phosphatase (EC 3.1.3.-) [GenBank Z38060 or U18813x11]; glucose-1-phosphatase (EC 3.1.3.10) [GenBank M33807]; glucose-6-phosphatase (EC 3.1.3.9) [GenBank U00445]; fructose-1,6-bisphosphatase (EC 3.1.3.11) [GenBank X12545 or J03207] or phosphatidyl glycerol phosphate phosphatase (EC 3.1.3.27) [GenBank M23546 and M23628].

Genes encoding glycerol kinase are known. For example, GUT1 encoding the glycerol kinase from *Saccharomyces* has been isolated and sequenced (Pavlik et al., *Curr. Genet.* 24, 21, (1993)) and the base sequence is given by SEQ ID NO:10 which encodes the amino acid sequence given in SEQ ID NO:18. It will be appreciated by the skilled artisan that although glycerol kinase catalyzes the degradation of glycerol in nature the same enzyme will be able to function in the synthesis of glycerol to convert glycerol-3-phosphate to glycerol under the appropriate reaction energy conditions. Evidence exists for glycerol production through a glycerol kinase. Under anaerobic or respiration-inhibited conditions, *Trypanosoma brucei* gives rise to glycerol in the presence of Glycerol-3-P and ADP. The reaction occurs in the glycosome compartment (D. Hammond, *J. Biol. Chem.* 260, 15646-15654, (1985)).

Host cells

Suitable host cells for the recombinant production of 1,3-propanediol may be either prokaryotic or eukaryotic and will be limited only by the host cell ability to express active enzymes. Preferred hosts will be those typically useful for production of glycerol or 1,3-propanediol such as *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*. Most preferred in the present invention are *E. coli*, *Klebsiella* species and *Saccharomyces* species.

Adenosyl-cobalamin (coenzyme B₁₂) is an essential cofactor for glycerol dehydratase activity. The coenzyme is the most complex non-polymeric natural product known, and its synthesis *in vivo* is directed using the products of about 30 genes. Synthesis of coenzyme B₁₂ is found in prokaryotes, some of which are able to synthesize the compound *de novo*, while others can perform partial reactions. *E. coli*, for example, cannot fabricate the corrin ring structure, but is able to catalyze the conversion of cobinamide to corrinoid and can introduce the 5'-deoxyadenosyl group.

Eukaryotes are unable to synthesize coenzyme B₁₂ *de novo* and instead transport vitamin B₁₂ from the extracellular milieu with subsequent conversion of the compound to its functional

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form of the compound by cellular enzymes. Three enzyme activities have been described for this series of reactions. 1) aquacobalamin reductase (EC 1.6.99.8) reduces Co(III) to Co(II); 2) cob(II)alamin reductase (EC 1.6.99.9) reduces Co(II) to Co(I); and 3) cob(I)alamin adenosyltransferase (EC 2.5.1.17) transfers a 5'deoxyadenosine moiety from ATP to the reduced

5 corrinoid. This last enzyme activity is the best characterized of the three, and is encoded by *cobA* in *S. typhimurium*, *btuR* in *E. coli* and *cobO* in *P. denitrificans*. These three cob(I)alamin adenosyltransferase genes have been cloned and sequenced. Cob(I)alamin adenosyltransferase activity has been detected in human fibroblasts and in isolated rat mitochondria (Fenton et al., *Biochem. Biophys. Res. Commun.* 98, 283-9, (1981)). The two enzymes involved in cobalt

10 reduction are poorly characterized and gene sequences are not available. There are reports of an aquacobalamin reductase from *Euglena gracilis* (Watanabe et al., *Arch. Biochem. Biophys.* 305, 421-7, (1993)) and a microsomal cob(III)alamin reductase is present in the microsomal and mitochondrial inner membrane fractions from rat fibroblasts (Pezacka, *Biochim. Biophys. Acta*, 1157, 167-77, (1993)).

15 Supplementing culture media with vitamin B₁₂ may satisfy the need to produce coenzyme B₁₂ for glycerol dehydratase activity in many microorganisms, but in some cases additional catalytic activities may have to be added or increased *in vivo*. Enhanced synthesis of coenzyme B₁₂ in eukaryotes may be particularly desirable. Given the published sequences for genes encoding cob(I)alamin adenosyltransferase, the cloning and expression of this gene could be

20 accomplished by one skilled in the art. For example, it is contemplated that yeast, such as *Saccharomyces*, could be constructed so as to contain genes encoding cob(I)alamin adenosyltransferase in addition to the genes necessary to effect conversion of a carbon substrate such as glucose to 1,3-propanediol. Cloning and expression of the genes for cobalt reduction requires a different approach. This could be based on a selection in *E. coli* for growth on

25 ethanolamine as sole N₂ source. In the presence of coenzyme B₁₂ ethanolamine ammonia-lyase enables growth of cells in the absence of other N₂ sources. If *E. coli* cells contain a cloned gene for cob(I)alamin adenosyltransferase and random cloned DNA from another organism, growth on ethanolamine in the presence of aquacobalamin should be enhanced and selected for if the random cloned DNA encodes cobalt reduction properties to facilitate adenosylation of

30 aquacobalamin.

Glycerol dehydratase is a multi-subunit enzyme consisting of three protein components which are arranged in an $\alpha_2\beta_2\gamma_2$ configuration (M. Seyfried et al, *J. Bacteriol.*, 5793-5796 (1996)). This configuration is an inactive apo-enzyme which binds one molecule of coenzyme B₁₂ to become the catalytically active holo-enzyme. During catalysis, the holo-enzyme undergoes rapid,

35 first order inactivation, to become an inactive complex in which the coenzyme B₁₂ has been converted to hydroxycobalamin (Z. Schneider and J. Pawelkiewicz, *ACTA Biochim. Pol.* 311-328

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(1966)). Stoichiometric analysis of the reaction of glycerol dehydratase with glycerol as substrate revealed that each molecule of enzyme catalyzes 100,000 reactions before inactivation (Z. Schneider and J. Pawelkiewicz, ACTA Biochim. Pol. 311-328 (1966)). In vitro, this inactive complex can only be reactivated by removal of the hydroxycobalamin, by strong chemical treatment with magnesium and sulfite, and replacement with additional coenzyme B₁₂ (Z. Schneider et al., J. Biol. Chem. 3388-3396 (1970)). Inactivated glycerol dehydratase in wild type Klebsiella pneumoniae can be reactivated in situ (toluenized cells) in the presence of coenzyme B₁₂, adenosine 5'-triphosphate (ATP), and manganese (S. Honda et al, J. Bacteriol. 1458-1465 (1980)). This reactivation was shown to be due to the ATP dependent replacement of the inactivated cobalamin with coenzyme B₁₂ (K. Ushio et al., J. Nutr. Sci. Vitaminol. 225-236 (1982)). Cell extract from toluenized cells which in situ catalyze the ATP, manganese, and coenzyme B₁₂ dependent reactivation are inactive with respect to this reactivation. Thus, without strong chemical reductive treatment or cell mediated replacement of the inactivated cofactor, glycerol dehydratase can only catalyzed 100,000 reactions per molecule.

The present invention demonstrates that the presence of protein X is important for in vivo reactivation of the dehydratase and the production of 1,3-propanediol is increased in a host cell capable of producing 1,3-propanediol in the presence of protein X. The present invention also discloses that the presence of protein 1, protein 2 and protein 3, in combination with protein X, also increased the production of 1,3-propanediol in a host cell capable of producing 1,3-propanediol.

In addition to *E. coli* and *Saccharomyces*, *Klebsiella* is a particularly preferred host. Strains of *Klebsiella pneumoniae* are known to produce 1,3-propanediol when grown on glycerol as the sole carbon. It is contemplated that *Klebsiella* can be genetically altered to produce 1,3-propanediol from monosaccharides, oligosaccharides, polysaccharides, or one-carbon substrates.

In order to engineer such strains, it will be advantageous to provide the *Klebsiella* host with the genes facilitating conversion of dihydroxyacetone phosphate to glycerol and conversion of glycerol to 1,3-propanediol either separately or together, under the transcriptional control of one or more constitutive or inducible promoters. The introduction of the DAR1 and GPP2 genes encoding glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase, respectively, will provide *Klebsiella* with genetic machinery to produce 1,3-propanediol from an appropriate carbon substrate.

The genes encoding protein X, protein 1, protein 2 and protein 3 or other enzymes associated with 1,3-propanediol production (e.g., G3PDH, G3P phosphatase, *dhaB* and/or *dhaT*) may be introduced on any plasmid vector capable of replication in *K. pneumoniae* or they may be integrated into the *K. pneumoniae* genome. For example, *K. pneumoniae* ATCC 25955 and

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K. pneumoniae ECL 2106 are known to be sensitive to tetracycline or chloramphenicol; thus plasmid vectors which are both capable of replicating in *K. pneumoniae* and encoding resistance to either or both of these antibiotics may be used to introduce these genes into *K. pneumoniae*. Methods of transforming *Klebsiella* with genes of interest are common and well known in the art and suitable protocols, including appropriate vectors and expression techniques may be found in Sambrook, *supra*.

Vectors and expression cassettes

The present invention provides a variety of vectors and transformation and expression cassettes suitable for the cloning, transformation and expression of protein X, protein 1, protein 2 and protein 3 as well as other proteins associated with 1,3-propanediol production, e.g., G3PDH and G3P phosphatase into a suitable host cell. Suitable vectors will be those which are compatible with the bacterium employed. Suitable vectors can be derived, for example, from a bacteria, a virus (such as bacteriophage T7 or a M-13 derived phage), a cosmid, a yeast or a plant. Protocols for obtaining and using such vectors are known to those in the art. (Sambrook et al., *Molecular Cloning: A Laboratory Manual* - volumes 1,2,3 (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, (1989)).

Typically, the vector or cassette contains sequences directing transcription and translation of the relevant gene, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. It is most preferred when both control regions are derived from genes homologous to the transformed host cell although it is to be understood that such control regions need not be derived from the genes native to the specific species chosen as a production host.

Initiation control regions or promoters, which are useful to drive expression of the protein x and protein 1, protein 2 or protein 3 in the desired host cell, are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these genes is suitable for the present invention including but not limited to CYC1, HIS3, GAL1, GAL10, ADH1, PGK, PHO5, GAPDH, ADC1, TRP1, URA3, LEU2, ENO, TPI (useful for expression in *Saccharomyces*); AOX1 (useful for expression in *Pichia*); and lac, trp, IP_L , IP_R , T7, tac, and trc (useful for expression in *E. coli*).

Termination control regions may also be derived from various genes native to the preferred hosts. Optionally, a termination site may be unnecessary, however, it is most preferred if included.

For effective expression of the instant enzymes, DNA encoding the enzymes are linked operably through initiation codons to selected expression control regions such that expression results in the formation of the appropriate messenger RNA.

Transformation of suitable hosts and expression of genes for the production of 1,3-propanediol

Once suitable cassettes are constructed they are used to transform appropriate host cells. Introduction of the cassette containing *dhaB* activity, *dhaB* protein X and at least one of protein 1, protein 2 and protein 3 and optionally 1,3-propanediol oxidoreductase (*dhaT*), either separately or together, into the host cell may be accomplished by known procedures such as by transformation (e.g., using calcium-permeabilized cells, electroporation) or by transfection using a recombinant phage virus. (Sambrook et al., *supra.*). In the present invention, *E. coli* DH5a was transformed with *dhaB* subunits 1, 2 and 3 and *dha* protein X.

Additionally, *E. coli* W2042 (ATCC 98188) containing the genes encoding glycerol-3-phosphate dehydrogenase (G3PDH) and glycerol-3-phosphatase (G3P phosphatase), glycerol dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase (*dhaT*) was created. Additionally, *S. cerevisiae* YPH500 (ATCC 74392) harboring plasmids pMCK10, pMCK17, pMCK30 and pMCK35 containing genes encoding glycerol-3-phosphate dehydrogenase (G3PDH) and glycerol-3-phosphatase (G3P phosphatase), glycerol dehydratase (*dhaB*), and 1,3-propanediol oxidoreductase (*dhaT*) was constructed. Both the above-mentioned transformed *E. coli* and *Saccharomyces* represent preferred embodiments of the invention.

Media and Carbon Substrates:

Fermentation media in the present invention must contain suitable carbon substrates.

Suitable substrates may include but are not limited to monosaccharides such as glucose and fructose, oligosaccharides such as lactose or sucrose, polysaccharides such as starch or cellulose, or mixtures thereof, and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. Additionally, the carbon substrate may also be one-carbon substrates such as carbon dioxide, or methanol for which metabolic conversion into key biochemical intermediates has been demonstrated. Glycerol production from single carbon sources (e.g., methanol, formaldehyde, or formate) has been reported in methylotrophic yeasts (Yamada et al., *Agric. Biol. Chem.*, 53(2) 541-543, (1989)) and in bacteria (Hunter et.al., *Biochemistry*, 24, 4148-4155, (1985)). These organisms can assimilate single carbon compounds, ranging in oxidation state from methane to formate, and produce glycerol. The pathway of carbon assimilation can be through ribulose monophosphate, through serine, or through xylulose-momophosphate (Gottschalk, Bacterial Metabolism, Second Edition, Springer-Verlag: New York (1986)). The ribulose monophosphate pathway involves the condensation of formate with ribulose-5-phosphate to form a 6 carbon sugar that becomes fructose and eventually the three carbon product glyceraldehyde-3-phosphate. Likewise, the serine pathway assimilates the one-carbon compound into the glycolytic pathway via methylenetetrahydrofolate.

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In addition to utilization of one and two carbon substrates, methylotrophic organisms are also known to utilize a number of other carbon-containing compounds such as methylamine, glucosamine and a variety of amino acids for metabolic activity. For example, methylotrophic yeast are known to utilize the carbon from methylamine to form trehalose or glycerol (Bellion et al., *Microb. Growth C1 Compd.*, [Int. Symp.], 7th (1993), 415-32. Editor(s): Murrell, J. Collin; Kelly, Don P. Publisher: Intercept, Andover, UK). Similarly, various species of *Candida* will metabolize alanine or oleic acid (Sulter et al., *Arch. Microbiol.*, 153(5), 485-9 (1990)). Hence, the source of carbon utilized in the present invention may encompass a wide variety of carbon-containing substrates and will only be limited by the requirements of the host organism.

Although it is contemplated that all of the above mentioned carbon substrates and mixtures thereof are suitable in the present invention, preferred carbon substrates are monosaccharides, oligosaccharides, polysaccharides, and one-carbon substrates. More preferred are sugars such as glucose, fructose, sucrose and single carbon substrates such as methanol and carbon dioxide. Most preferred is glucose.

In addition to an appropriate carbon source, fermentation media must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for the growth of the cultures and promotion of the enzymatic pathway necessary for glycerol production. Particular attention is given to Co(II) salts and/or vitamin B₁₂ or precursors thereof.

Culture Conditions:

Typically, cells are grown at 30 °C in appropriate media. Preferred growth media in the present invention are common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth or Yeast Malt Extract (YM) broth. Other defined or synthetic growth media may also be used and the appropriate medium for growth of the particular microorganism will be known by someone skilled in the art of microbiology or fermentation science. The use of agents known to modulate catabolite repression directly or indirectly, e.g., cyclic adenosine 2':3'-monophosphate or cyclic adenosine 2':5'-monophosphate, may also be incorporated into the reaction media. Similarly, the use of agents known to modulate enzymatic activities (e.g., sulphites, bisulphites and alkalis) that lead to enhancement of glycerol production may be used in conjunction with or as an alternative to genetic manipulations.

Suitable pH ranges for the fermentation are between pH 5.0 to pH 9.0, where pH 6.0 to pH 8.0 is preferred as range for the initial condition.

Reactions may be performed under aerobic or anaerobic conditions where anaerobic or microaerobic conditions are preferred.

Batch and Continuous Fermentations:

The present process uses a batch method of fermentation. A classical batch fermentation is a closed system where the composition of the media is set at the beginning of the fermentation and not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the media is inoculated with the desired organism or organisms and fermentation is permitted to occur adding nothing to the system. Typically, however, a batch fermentation is "batch" with respect to the addition of the carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. The metabolite and biomass compositions of the batch system change constantly up to the time the fermentation is stopped. Within batch cultures cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase will eventually die. Cells in log phase generally are responsible for the bulk of production of end product or intermediate.

A variation on the standard batch system is the Fed-Batch fermentation system which is also suitable in the present invention. In this variation of a typical batch system, the substrate is added in increments as the fermentation progresses. Fed-Batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the media. Measurement of the actual substrate concentration in Fed-Batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO₂. Batch and Fed-Batch fermentations are common and well known in the art and examples may be found in Brock, *supra*.

It is also contemplated that the method would be adaptable to continuous fermentation methods. Continuous fermentation is an open system where a defined fermentation media is added continuously to a bioreactor and an equal amount of conditioned media is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, one method will maintain a limiting nutrient such as the carbon source or nitrogen level at a fixed rate and allow all other parameters to moderate. In other systems a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions and thus the cell loss due to media being drawn off must be balanced against the cell growth rate in the fermentation.

Methods of modulating nutrients and growth factors for continuous fermentation processes as

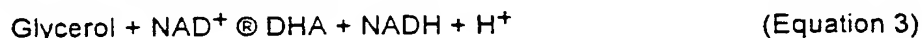
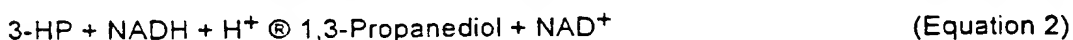
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well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, *supra*.

The present invention may be practiced using either batch, fed-batch or continuous processes and that any known mode of fermentation would be suitable. Additionally, it is contemplated that cells may be immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for 1,3-propanediol production.

Alterations in the 1,3-propanediol production pathway:

Representative enzyme pathway. The production of 1,3-propanediol from glucose can be accomplished by the following series of steps. This series is representative of a number of pathways known to those skilled in the art. Glucose is converted in a series of steps by enzymes of the glycolytic pathway to dihydroxyacetone phosphate (DHAP) and 3-phosphoglyceraldehyde (3-PG). Glycerol is then formed by either hydrolysis of DHAP to dihydroxyacetone (DHA) followed by reduction, or reduction of DHAP to glycerol 3-phosphate (G3P) followed by hydrolysis. The hydrolysis step can be catalyzed by any number of cellular phosphatases which are known to be specific or non-specific with respect to their substrates or the activity can be introduced into the host by recombination. The reduction step can be catalyzed by a NAD⁺ (or NADP⁺) linked host enzyme or the activity can be introduced into the host by recombination. It is notable that the *dha* regulon contains a glycerol dehydrogenase (E.C. 1.1.1.6) which catalyzes the reversible reaction of Equation 3.



Glycerol is converted to 1,3-propanediol via the intermediate 3-hydroxypropionaldehyde (3-HP) as has been described in detail above. The intermediate 3-HP is produced from glycerol (Equation 1) by a dehydratase enzyme which can be encoded by the host or can be introduced into the host by recombination. This dehydratase can be glycerol dehydratase (E.C. 4.2.1.30), diol dehydratase (E.C. 4.2.1.28), or any other enzyme able to catalyze this transformation. Glycerol dehydratase, but not diol dehydratase, is encoded by the *dha* regulon. 1,3-Propanediol is produced from 3-HP (Equation 2) by a NAD⁺- (or NADP⁺) linked host enzyme or the activity can be introduced into the host by recombination. This final reaction in the production of 1,3-propanediol can be catalyzed by 1,3-propanediol dehydrogenase (E.C. 1.1.1.202) or other alcohol dehydrogenases.

Mutations and transformations that affect carbon channeling. A variety of mutant organisms comprising variations in the 1,3-propanediol production pathway will be useful in the present

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invention. The introduction of a triosephosphate isomerase mutation (*tpi*-) into the microorganism is an example of the use of a mutation to improve the performance by carbon channeling. Alternatively, mutations which diminish the production of ethanol (*adh*) or lactate (*ldh*) will increase the availability of NADH for the production of 1,3-propanediol. Additional mutations in steps of glycolysis after glyceraldehyde-3-phosphate such as phosphoglycerate mutase (*pgm*) would be useful to increase the flow of carbon to the 1,3-propanediol production pathway. Mutations that effect glucose transport such as PTS which would prevent loss of PEP may also prove useful. Mutations which block alternate pathways for intermediates of the 1,3-propanediol production pathway such as the glycerol catabolic pathway (*g/p*) would also be useful to the present invention. The mutation can be directed toward a structural gene so as to impair or improve the activity of an enzymatic activity or can be directed toward a regulatory gene so as to modulate the expression level of an enzymatic activity.

Alternatively, transformations and mutations can be combined so as to control particular enzyme activities for the enhancement of 1,3-propanediol production. Thus it is within the scope of the present invention to anticipate modifications of a whole cell catalyst which lead to an increased production of 1,3-propanediol.

Identification and purification of 1,3-propanediol:

Methods for the purification of 1,3-propanediol from fermentation media are known in the art. For example, propanediols can be obtained from cell media by subjecting the reaction mixture to extraction with an organic solvent, distillation and column chromatography (U.S. 5,356,812). A particularly good organic solvent for this process is cyclohexane (U.S. 5,008,473).

1,3-Propanediol may be identified directly by submitting the media to high pressure liquid chromatography (HPLC) analysis. Preferred in the present invention is a method where fermentation media is analyzed on an analytical ion exchange column using a mobile phase of 0.01 N sulfuric acid in an isocratic fashion.

Identification and purification of G3PDH and G3P phosphatase:

The levels of expression of the proteins G3PDH and G3P phosphatase are measured by enzyme assays, G3PDH activity assay relied on the spectral properties of the cosubstrate, NADH, in the DHAP conversion to G-3-P. NADH has intrinsic UV/vis absorption and its consumption can be monitored spectrophotometrically at 340 nm. G3P phosphatase activity can be measured by any method of measuring the inorganic phosphate liberated in the reaction. The most commonly used detection method used the visible spectroscopic determination of a blue-colored phosphomolybdate ammonium complex.

EXAMPLES

GENERAL METHODS

Procedures for phosphorylations, ligations and transformations are well known in the art. Techniques suitable for use in the following examples may be found in Sambrook, J. et al.,
5 Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).

Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found as set out in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N.
10 Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994)) or by Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition, Sinauer Associates, Inc., Sunderland, MA (1989). All reagents and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories
15 (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "mL" means milliliters, "L" means liters.

ENZYME ASSAYS

20 Glycerol dehydratase activity in cell-free extracts was determined using 1,2-propanediol as substrate. The assay, based on the reaction of aldehydes with methylbenzo-2-thiazolone hydrazone, has been described by Forage and Foster (*Biochim. Biophys. Acta*, 569, 249 (1979)). The activity of 1,3-propanediol oxidoreductase, sometimes referred to as 1,3-propanediol dehydrogenase, was determined in solution or in slab gels using 1,3-propanediol and NAD⁺ as
25 substrates as has also been described. Johnson and Lin, *J. Bacteriol.*, 169, 2050 (1987). NADH or NADPH dependent glycerol 3-phosphate dehydrogenase (G3PDH) activity was determined spectrophotometrically, following the disappearance of NADH or NADPH as has been described. (R. M. Bell and J. E. Cronan, Jr., *J. Biol. Chem.* 250:7153-8 (1975)).

Honda et al. (1980, In Situ Reactivation of Glycerol-Inactivated Coenzyme B₁₂-Dependent
30 Enzymes, Glycerol Dehydratase and Diol Dehydratase. *Journal of Bacteriology* 143:1458-1465) disclose an assay that measures the reactivation of dehydratases.

Assay for glycerol-3-phosphatase, GPP

The assay for enzyme activity was performed by incubating the extract with an organic phosphate substrate in a bis-Tris or MES and magnesium buffer, pH 6.5. The substrate used
35 was l-a-glycerol phosphate; d,l-a-glycerol phosphate. The final concentrations of the reagents in the assay are: buffer (20 mM, bis-Tris or 50 mM MES); MgCl₂ (10 mM); and substrate (20 mM).

If the total protein in the sample was low and no visible precipitation occurs with an acid quench, the sample was conveniently assayed in the cuvette. This method involved incubating an enzyme sample in a cuvette that contained 20 mM substrate (50 mM, 200 mM), 50 mM MES, 10 mM MgCl_2 , pH 6.5 buffer. The final phosphatase assay volume was 0.5 mL. The enzyme-containing sample was added to the reaction mixture; the contents of the cuvette were mixed and then the cuvette was placed in a circulating water bath at $T = 37^\circ\text{C}$ for 5 to 120 min -- depending on whether the phosphatase activity in the enzyme sample ranged from 2 to 0.02 U/mL. The enzymatic reaction was quenched by the addition of the acid molybdate reagent (0.4 mL). After the Fiske SubbaRow reagent (0.1 mL) and distilled water (1.5 mL) were added, the solution was mixed and allowed to develop. After 10 min, the absorbance of the samples was read at 660 nm using a Cary 219 UV/Vis spectrophotometer. The amount of inorganic phosphate released was compared to a standard curve that was prepared by using a stock inorganic phosphate solution (0.65 mM) and preparing 6 standards with final inorganic phosphate concentrations ranging from 0.026 to 0.130 mmol/mL.

Isolation and Identification 1,3-propanediol

The conversion of glycerol to 1,3-propanediol was monitored by HPLC. Analyses were performed using standard techniques and materials available to one skilled in the art of chromatography. One suitable method utilized a Waters Maxima 820 HPLC system using UV (210 nm) and RI detection. Samples were injected onto a Shodex SH-1011 column (8 mm x 300 mm, purchased from Waters, Milford, MA) equipped with a Shodex SH-1011P precolumn (6 mm x 50 mm), temperature controlled at 50°C , using 0.01 N H_2SO_4 as mobile phase at a flow rate of 0.5 mL/min. When quantitative analysis was desired, samples were prepared with a known amount of trimethylacetic acid as external standard. Typically, the retention times of glycerol (RI detection), 1,3-propanediol (RI detection), and trimethylacetic acid (UV and RI detection) were 20.67 min, 26.08 min, and 35.03 min, respectively.

Production of 1,3-propanediol was confirmed by GC/MS. Analyses were performed using standard techniques and materials available to one of skill in the art of GC/MS. One suitable method utilized a Hewlett Packard 5890 Series II gas chromatograph coupled to a Hewlett Packard 5971 Series mass selective detector (EI) and a HP-INNOWax column (30 m length, 0.25 mm i.d., 0.25 micron film thickness). The retention time and mass spectrum of 1,3-propanediol generated were compared to that of authentic 1,3-propanediol (m/e : 57, 58).

An alternative method for GC/MS involved derivatization of the sample. To 1.0 mL of sample (e.g., culture supernatant) was added 30 μL of concentrated (70% v/v) perchloric acid. After mixing, the sample was frozen and lyophilized. A 1:1 mixture of bis(trimethylsilyl)trifluoroacetamide:pyridine (300 μL) was added to the lyophilized material, mixed vigorously and placed at 65°C for one h. The sample was clarified of insoluble material by

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centrifugation. The resulting liquid partitioned into two phases, the upper of which was used for analysis. The sample was chromatographed on a DB-5 column (48 m, 0.25 mm I.D., 0.25 μ m film thickness; from J&W Scientific) and the retention time and mass spectrum of the 1,3-propanediol derivative obtained from culture supernatants were compared to that obtained from authentic standards. The mass spectrum of TMS-derivatized 1,3-propanediol contains the characteristic ions of 205, 177, 130 and 115 AMU.

EXAMPLE 1

CLONING AND TRANSFORMATION OF *E. COLI* HOST CELLS WITH COSMID DNA FOR THE EXPRESSION OF 1,3-PROPANEDIOL

Media

Synthetic S12 medium was used in the screening of bacterial transformants for the ability to make 1,3-propanediol. S12 medium contains: 10 mM ammonium sulfate, 50 mM potassium phosphate buffer, pH 7.0, 2 mM $MgCl_2$, 0.7 mM $CaCl_2$, 50 μ M $MnCl_2$, 1 μ M $FeCl_3$, 1 μ M $ZnCl_2$, 1.7 μ M $CuSO_4$, 2.5 μ M $CoCl_2$, 2.4 μ M Na_2MoO_4 , and 2 μ M thiamine hydrochloride.

Medium A used for growth and fermentation consisted of: 10 mM ammonium sulfate; 50 mM MOPS/KOH buffer, pH 7.5; 5 mM potassium phosphate buffer, pH 7.5; 2 mM $MgCl_2$; 0.7 mM $CaCl_2$; 50 μ M $MnCl_2$; 1 μ M $FeCl_3$; 1 μ M $ZnCl_2$; 1.72 μ M $CuSO_4$; 2.53 μ M $CoCl_2$; 2.42 μ M Na_2MoO_4 ; 2 μ M thiamine hydrochloride; 0.01% yeast extract; 0.01% casamino acids; 0.8 μ g/mL vitamin B₁₂; and 50 μ g/mL amp. Medium A was supplemented with either 0.2% glycerol or 0.2% glycerol plus 0.2% D-glucose as required.

Cells:

Klebsiella pneumoniae ECL2106 (Ruch et al., *J. Bacteriol.*, 124, 348 (1975)), also known in the literature as *K. aerogenes* or *Aerobacter aerogenes*, was obtained from E. C. C. Lin (Harvard Medical School, Cambridge, MA) and was maintained as a laboratory culture.

Klebsiella pneumoniae ATCC 25955 was purchased from American Type Culture Collection (Rockville, MD).

E. coli DH5a was purchased from Gibco/BRL and was transformed with the cosmid DNA isolated from *Klebsiella pneumoniae* ATCC 25955 containing a gene coding for either a glycerol or diol dehydratase enzyme. Cosmids containing the glycerol dehydratase were identified as pKP1 and pKP2 and cosmid containing the diol dehydratase enzyme were identified as pKP4. Transformed DH5a cells were identified as DH5a-pKP1, DH5a-pKP2, and DH5a-pKP4.

E. coli ECL707 (Sprenger et al., *J. Gen. Microbiol.*, 135, 1255 (1989)) was obtained from E. C. C. Lin (Harvard Medical School, Cambridge, MA) and was similarly transformed with cosmid DNA from *Klebsiella pneumoniae*. These transformants were identified as ECL707-pKP1 and ECL707-pKP2, containing the glycerol dehydratase gene and ECL707-pKP4 containing the diol dehydratase gene.

E. coli AA200 containing a mutation in the *tpi* gene (Anderson et al., *J. Gen Microbiol.*, 62, 329 (1970)) was purchased from the *E. coli* Genetic Stock Center, Yale University (New Haven, CT) and was transformed with *Klebsiella* cosmid DNA to give the recombinant organisms AA200-pKP1 and AA200-pKP2, containing the glycerol dehydratase gene, and AA200-pKP4, containing the diol dehydratase gene.

DH5a:

Six transformation plates containing approximately 1,000 colonies of *E. coli* XL1-Blue MR transfected with *K. pneumoniae* DNA were washed with 5 mL LB medium and centrifuged. The bacteria were pelleted and resuspended in 5 mL LB medium + glycerol. An aliquot (50 uL) was inoculated into a 15 mL tube containing S12 synthetic medium with 0.2% glycerol + 400 ng per mL of vitamin B₁₂ + 0.001% yeast extract + 50amp. The tube was filled with the medium to the top and wrapped with parafilm and incubated at 30 °C. A slight turbidity was observed after 48 h. Aliquots, analyzed for product distribution as described above at 78 h and 132 h, were positive for 1,3-propanediol, the later time points containing increased amounts of 1,3-propanediol.

The bacteria, testing positive for 1,3-propanediol production, were serially diluted and plated onto LB-50amp plates in order to isolate single colonies. Forty-eight single colonies were isolated and checked again for the production of 1,3-propanediol. Cosmid DNA was isolated from 6 independent clones and transformed into *E. coli* strain DH5a. The transformants were again checked for the production of 1,3-propanediol. Two transformants were characterized further and designated as DH5a-pKP1 and DH5a-pKP2.

A 12.1 kb EcoRI-Sall fragment from pKP1, subcloned into pIBI31 (IBI Biosystem, New Haven, CT), was sequenced and termed pHK28-26 (SEQ ID NO:19). Sequencing revealed the loci of the relevant open reading frames of the *dha* operon encoding glycerol dehydratase and genes necessary for regulation. Referring to SEQ ID NO:19, a fragment of the open reading frame for *dhaK* encoding dihydroxyacetone kinase is found at bases 1-399; the open reading frame *dhaD* encoding glycerol dehydrogenase is found at bases 983-2107; the open reading frame *dhaR* encoding the repressor is found at bases 2209-4134; the open reading frame *dhaT* encoding 1,3-propanediol oxidoreductase is found at bases 5017-6180; the open reading frame *dhaB1* encoding the alpha subunit glycerol dehydratase is found at bases 7044-8711; the open reading frame *dhaB2* encoding the beta subunit glycerol dehydratase is found at bases 8724-9308; the open reading frame *dhaB3* encoding the gamma subunit glycerol dehydratase is found at bases 9311-9736; and the open reading frame *dhaBX*, encoding a protein of unknown function is found at bases 9749-11572.

Single colonies of *E. coli* XL1-Blue MR transfected with packaged cosmid DNA from *K. pneumoniae* were inoculated into microtiter wells containing 200 uL of S15 medium (ammonium sulfate, 10 mM; potassium phosphate buffer, pH 7.0, 1 mM; MOPS/KOH buffer,

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pH 7.0, 50 mM; MgCl₂, 2 mM; CaCl₂, 0.7 mM; MnCl₂, 50 μ M; FeCl₃, 1 μ M; ZnCl, 1 μ M; CuSO₄, 1.72 μ M; CoCl₂, 2.53 μ M; Na₂MoO₄, 2.42 μ M; and thiamine hydrochloride, 2 μ M) + 0.2% glycerol + 400 ng/mL of vitamin B₁₂ + 0.001% yeast extract + 50 μ g/mL ampicillin. In addition to the microtiter wells, a master plate containing LB-50 amp was also inoculated. After 96 h, 100 μ L was withdrawn and centrifuged in a Rainin microfuge tube containing a 0.2 micron nylon membrane filter. Bacteria were retained and the filtrate was processed for HPLC analysis. Positive clones demonstrating 1,3-propanediol production were identified after screening approximately 240 colonies. Three positive clones were identified, two of which had grown on LB-50 amp and one of which had not. A single colony, isolated from one of the two positive clones grown on LB-50 amp and verified for the production of 1,3-propanediol, was designated as pKP4. Cosmid DNA was isolated from *E. coli* strains containing pKP4 and *E. coli* strain DH5a was transformed. An independent transformant, designated as DH5a-pKP4, was verified for the production of 1,3-propanediol.

ECL707:

E. coli strain ECL707 was transformed with cosmid *K. pneumoniae* DNA corresponding to one of pKP1, pKP2, pKP4 or the Supercos vector alone and named ECL707-pKP1, ECL707-pKP2, ECL707-pKP4, and ECL707-sc, respectively. ECL707 is defective in *glpK*, *gld*, and *ptsD* which encode the ATP-dependent glycerol kinase, NAD⁺-linked glycerol dehydrogenase, and enzyme II for dihydroxyacetone of the phosphoenolpyruvate-dependent phosphotransferase system, respectively.

Twenty single colonies of each cosmid transformation and five of the Supercos vector alone (negative control) transformation, isolated from LB-50amp plates, were transferred to a master LB-50amp plate. These isolates were also tested for their ability to convert glycerol to 1,3-propanediol in order to determine if they contained dehydratase activity. The transformants were transferred with a sterile toothpick to microtiter plates containing 200 μ L of Medium A supplemented with either 0.2% glycerol or 0.2% glycerol plus 0.2% D-glucose. After incubation for 48 hr at 30 °C, the contents of the microtiter plate wells were filtered through an 0.45 micron nylon filter and chromatographed by HPLC. The results of these tests are given in Table 1.

Table 1

Conversion of glycerol to 1,3-propanediol by transformed ECL707

<u>Transformant</u>	<u>Glycerol*</u>	<u>Glycerol plus Glucose*</u>
ECL707-pKP1	19/20	19/20
ECL707-pKP2	18/20	20/20
ECL707-pKP4	0/20	20/20
ECL707-sc	0/5	0/5

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*(Number of positive isolates/number of isolates tested)

AA200:

E. coli strain AA200 was transformed with cosmid *K. pneumoniae* DNA corresponding to one of pKP1, pKP2, pKP4 and the Supercos vector alone and named AA200-pKP1, AA200-pKP2, AA200-pKP4, and AA200-sc, respectively. Strain AA200 is defective in triosephosphate isomerase (*tpi*⁻).

Twenty single colonies of each cosmid transformation and five of the empty vector transformation were isolated and tested for their ability to convert glycerol to 1,3-propanediol as described for *E. coli* strain ECL707. The results of these tests are given in Table 2.

Table 2

Conversion of glycerol to 1,3-propanediol by transformed AA200

<u>Transformant</u>	<u>Glycerol*</u>	<u>Glycerol plus Glucose*</u>
AA200-pKP1	17/20	17/20
AA200-pKP2	17/20	17/20
AA200-pKP4	2/20	16/20
AA200-sc	0/5	0/5

*(Number of positive isolates/number of isolates tested)

EXAMPLE 2

CONVERSION OF D-GLUCOSE TO 1,3-PROPANEDIOL BY RECOMBINANT *E. coli* USING

DAR1, GPP2, *dhaB*, and *dhaT*

Construction of general purpose expression plasmids for use in transformation of *Escherichia coli*

The expression vector pTacIQ

The *E. coli* expression vector, pTacIQ, contains the *lacIq* gene (Farabaugh, *Nature* 274, 5673 (1978)) and *tac* promoter (Amann et al., *Gene* 25, 167 (1983)) inserted into the *EcoRI* of pBR322 (Sutcliffe et al., *Cold Spring Harb. Symp. Quant. Biol.* 43, 77 (1979)). A multiple cloning site and terminator sequence (SEQ ID NO:20) replaces the pBR322 sequence from *EcoRI* to *SphI*.

Subcloning the glycerol dehydratase genes (*dhaB1*, 2, 3)

The open reading frame for *dhaB3* gene (incorporating an *EcoRI* site at the 5' end and a *XbaI* site at the 3' end) was amplified from pHK28-26 by PCR using primers (SEQ ID NOS:21 and 22). The product was subcloned into pLitmus29 (New England Biolab, Inc., Beverly, MA) to generate the plasmid pDHAB3 containing *dhaB3*.

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The region containing the entire coding region for the four genes of the *dhaB* operon from pHK28-26 was cloned into pBluescriptII KS+ (Stratagene, La Jolla, CA) using the restriction enzymes KpnI and EcoRI to create the plasmid pM7.

The *dhaBX* gene was removed by digesting the plasmid pM7, which contains
5 *dhaB*(1,2,3,4), with Apal and XbaI (deleting part of *dhaB3* and all of *dhaBX*). The resulting 5.9 kb fragment was purified and ligated with the 325-bp Apal-XbaI fragment from plasmid pDHAB3 (restoring the *dhaB3* gene) to create pM11, which contains *dhaB*(1,2,3).

The open reading frame for the *dhaB1* gene (incorporating a HindIII site and a consensus RBS ribosome binding site at the 5' end and a XbaI site at the 3' end) was amplified from
10 pHK28-26 by PCR using primers (SEQ ID NO:23 and SEQ ID NO:24). The product was subcloned into pLitmus28 (New England Biolab, Inc.) to generate the plasmid pDT1 containing *dhaB1*.

A NotI-XbaI fragment from pM11 containing part of the *dhaB1* gene, the *dhaB2* gene and the *dhaB3* gene was inserted into pDT1 to create the *dhaB* expression plasmid, pDT2. The
15 HindIII-XbaI fragment containing the *dhaB*(1,2,3) genes from pDT2 was inserted into pTaciQ to create pDT3.

Subcloning the 1,3-propanediol dehydrogenase gene (*dhaT*)

The KpnI-SacI fragment of pHK28-26, containing the complete 1,3-propanediol dehydrogenase (*dhaT*) gene, was subcloned into pBluescriptII KS+ creating plasmid pAH1. The
20 *dhaT* gene (incorporating an XbaI site at the 5' end and a BamHI site at the 3' end) was amplified by PCR from pAH1 as template DNA using synthetic primers (SEQ ID NO:25 with SEQ ID NO:26). The product was subcloned into pCR-Script (Stratagene) at the SrfI site to generate the plasmids pAH4 and pAH5 containing *dhaT*. The plasmid pAH4 contains the *dhaT* gene in the correct orientation for expression from the lac promoter in pCR-Script and pAH5 contains the
25 *dhaT* gene in the opposite orientation. The XbaI-BamHI fragment from pAH4 containing the *dhaT* gene was inserted into pTaciQ to generate plasmid pAH8. The HindIII-BamHI fragment from pAH8 containing the RBS and *dhaT* gene was inserted into pBluescriptII KS+ to create pAH11. The HindIII-Sall fragment from pAH8 containing the RBS, *dhaT* gene and terminator was inserted into pBluescriptII SK+ to create pAH12.

Construction of an expression cassette for *dhaB*(1,2,3) and *dhaT*

An expression cassette for the *dhaB*(1,2,3) and *dhaT* was assembled from the individual *dhaB*(1,2,3) and *dhaT* subclones described above using standard molecular biology methods. The SpeI-KpnI fragment from pAH8 containing the RBS, *dhaT* gene and terminator was inserted into the XbaI-KpnI sites of pDT3 to create pAH23. The SmaI-EcoRI fragment between the *dhaB3*
35 and *dhaT* gene of pAH23 was removed to create pAH26. The SpeI-NotI fragment containing an EcoRI site from pDT2 was used to replace the SpeI-NotI fragment of pAH26 to generate pAH27.

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Construction of expression cassette for *dhaT* and *dhaB*(1,2,3)

An expression cassette for *dhaT* and *dhaB*(1,2,3) was assembled from the individual *dhaB*(1,2,3) and *dhaT* subclones described previously using standard molecular biology methods. A *SpeI*-*SacI* fragment containing the *dhaB*(1,2,3) genes from pDT3 was inserted into pAH11 at the *SpeI*-*SacI* sites to create pAH24.

Cloning and expression of glycerol 3-phosphatase for increased glycerol production in *E. coli*

The *Saccharomyces cerevisiae* chromosome V lamda clone 6592 (Gene Bank, accession # U18813x11) was obtained from ATCC. The glycerol 3- phosphate phosphatase (GPP2) gene (incorporating an *Bam*HI-RBS-*Xba*I site at the 5' end and a *Sma*I site at the 3' end) was cloned by PCR cloning from the lamda clone as target DNA using synthetic primers (SEQ ID NO:27 with SEQ ID NO:28). The product was subcloned into pCR-Script (Stratagene) at the *Srf*I site to generate the plasmids pAH15 containing GPP2. The plasmid pAH15 contains the GPP2 gene in the inactive orientation for expression from the lac promoter in pCR-Script SK+. The *Bam*HI-*Sma*I fragment from pAH15 containing the GPP2 gene was inserted into pBlueScriptII SK+ to generate plasmid pAH19. The pAH19 contains the GPP2 gene in the correct orientation for expression from the lac promoter. The *Xba*I-*Pst*II fragment from pAH19 containing the GPP2 gene was inserted into pPHOX2 to create plasmid pAH21.

Plasmids for the expression of *dhaT*, *dhaB*(1,2,3) and GPP2 genes

A *Sal*I-*Eco*RI-*Xba*I linker (SEQ ID NOS:29 and 30) was inserted into pAH5 which was digested with the restriction enzymes, *Sal*I-*Xba*I to create pDT16. The linker destroys the *Xba*I site. The 1 kb *Sal*I-*Mlu*I fragment from pDT16 was then inserted into pAH24 replacing the existing *Sal*I-*Mlu*I fragment to create pDT18.

The 4.1 kb *Eco*RI-*Xba*I fragment containing the expression cassette for *dhaT* and *dhaB*(1,2,3) from pDT18 and the 1.0 kb *Xba*I-*Sal*I fragment containing the GPP2 gene from pAH21 was inserted into the vector pMMB66EH (Füste et al., *GENE*, 48, 119 (1986)) digested with the restriction enzymes *Eco*RI and *Sal*I to create pDT20.

Plasmids for the over-expression of DAR1 in *E. coli*

DAR1 was isolated by PCR cloning from genomic *S. cerevisiae* DNA using synthetic primers (SEQ ID NO:46 with SEQ ID NO:47). Successful PCR cloning places an *Nco*I site at the 5' end of DAR1 where the ATG within *Nco*I is the DAR1 initiator methionine. At the 3' end of DAR1 a *Bam*HI site is introduced following the translation terminator. The PCR fragments were digested with *Nco*I + *Bam*HI and cloned into the same sites within the expression plasmid pTrc99A (Pharmacia, Piscataway, New Jersey) to give pDAR1A.

In order to create a better ribosome binding site at the 5' end of DAR1, a *Spe*I-RBS-*Nco*I linker obtained by annealing synthetic primers (SEQ ID NO:48 with SEQ ID NO:49) was inserted into the *Nco*I site of pDAR1A to create pAH40. Plasmid pAH40 contains the new RBS and DAR1

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gene in the correct orientation for expression from the trc promoter of Trc99A (Pharmacia). The NcoI-BamHI fragment from pDAR1A and a second set of SpeI-RBS-NcoI linker obtained by annealing synthetic primers (SEQ ID NO:31 with SEQ ID NO:32) was inserted into the SpeI-BamHI site of pBluescript II-SK+ (Stratagene) to create pAH41. The construct pAH41 contains an ampicillin resistance gene. The NcoI-BamHI fragment from pDAR1A and a second set of SpeI-RBS-NcoI linker obtained by annealing synthetic primers (SEQ ID NO:31 with SEQ ID NO:32) was inserted into the SpeI-BamHI site of pBC-SK+ (Stratagene) to create pAH42. The construct pAH42 contains a chloramphenicol resistance gene.

Construction of an expression cassette for DAR1 and GPP2

An expression cassette for DAR1 and GPP2 was assembled from the individual DAR1 and GPP2 subclones described above using standard molecular biology methods. The BamHI-PstI fragment from pAH19 containing the RBS and GPP2 gene was inserted into pAH40 to create pAH43. The BamHI-PstI fragment from pAH19 containing the RBS and GPP2 gene was inserted into pAH41 to create pAH44. The same BamHI-PstI fragment from pAH19 containing the RBS and GPP2 gene was also inserted into pAH42 to create pAH45.

The ribosome binding site at the 5' end of GPP2 was modified as follows. A BamHI-RBS-SpeI linker, obtained by annealing synthetic primers GATCCAGGAAACAGA with CTAGTCTGTTTCCTG to the XbaI-PstI fragment from pAH19 containing the GPP2 gene, was inserted into the BamHI-PstI site of pAH40 to create pAH48. Plasmid pAH48 contains the DAR1 gene, the modified RBS, and the GPP2 gene in the correct orientation for expression from the trc promoter of pTrc99A (Pharmacia, Piscataway, N.J.).

E. coli strain construction

E. coli W1485 is a wild-type K-12 strain (ATCC 12435). This strain was transformed with the plasmids pDT20 and pAH42 and selected on LA (Luria Agar, Difco) plates supplemented with 50 mg/mL carbencillim and 10 mg/mL chloramphenicol.

Production of 1,3-propanediol from glucose

E. coli W1485/pDT20/pAH42 was transferred from a plate to 50 mL of a medium containing per liter: 22.5 g glucose, 6.85 g K₂HPO₄, 6.3 g (NH₄)₂SO₄, 0.5 g NaHCO₃, 2.5 g NaCl, 8 g yeast extract, 8 g tryptone, 2.5 mg vitamin B₁₂, 2.5 mL modified Balch's trace-element solution, 50 mg carbencillim and 10 mg chloramphenicol, final pH 6.8 (HCl), then filter sterilized. The composition of modified Balch's trace-element solution can be found in Methods for General and Molecular Bacteriology (P. Gerhardt et al., eds, p. 158, American Society for Microbiology, Washington, DC (1994)). After incubating at 37 °C, 300 rpm for 6 h, 0.5 g glucose and IPTG (final concentration = 0.2 mM) were added and shaking was reduced to 100 rpm. Samples were analyzed by GC/MS. After 24 h, W1485/pDT20/pAH42 produced 1.1 g/L glycerol and 195 mg/L 1,3-propanediol.

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EXAMPLE 3CLONING AND EXPRESSION OF *dhaB* AND *dhaT*IN *Saccharomyces cerevisiae*

Expression plasmids that could exist as replicating episomal elements were constructed for each of the four *dha* genes. For all expression plasmids a yeast ADH1 promoter was present and separated from a yeast ADH1 transcription terminator by fragments of DNA containing recognition sites for one or more restriction endonucleases. Each expression plasmid also contained the gene for b-lactamase for selection in *E. coli* on media containing ampicillin, an origin of replication for plasmid maintenance in *E. coli*, and a 2 micron origin of replication for maintenance in *S. cerevisiae*. The selectable nutritional markers used for yeast and present on the expression plasmids were one of the following: HIS3 gene encoding imidazoleglycerolphosphate dehydratase, URA3 gene encoding orotidine 5'-phosphate decarboxylase, TRP1 gene encoding N-(5'-phosphoribosyl)-anthranilate isomerase, and LEU2 encoding b-isopropylmalate dehydrogenase.

The open reading frames for *dhaT*, *dhaB3*, *dhaB2* and *dhaB1* were amplified from pHK28-26 (SEQ ID NO:19) by PCR using primers (SEQ ID NO:38 with SEQ ID NO:39, SEQ ID NO:40 with SEQ ID NO:41, SEQ ID NO:42 with SEQ ID NO:43, and SEQ ID NO:44 with SEQ ID NO:45 for *dhaT*, *dhaB3*, *dhaB2* and *dhaB1*, respectively) incorporating EcoR1 sites at the 5' ends (10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.0001% gelatin, 200 mM dATP, 200 mM dCTP, 200 mM dGTP, 200 mM dTTP, 1 mM each primer, 1-10 ng target DNA, 25 units/mL Amplitaq[®] DNA polymerase (Perkin-Elmer Cetus, Norwalk CT)). PCR parameters were 1 min at 94 °C, 1 min at 55 °C; 1 min at 72 °C, 35 cycles. The products were subcloned into the EcoR1 site of pHIL-D4 (Phillips Petroleum, Bartlesville, OK) to generate the plasmids pMP13, pMP14, pMP20 and pMP15 containing *dhaT*, *dhaB3*, *dhaB2* and *dhaB1*, respectively.

Construction of *dhaB1* expression plasmid pMCK10

The 7.8 kb replicating plasmid pGADGH (Clontech, Palo Alto, CA) was digested with HindIII, dephosphorylated, and ligated to the *dhaB1* HindIII fragment from pMP15. The resulting plasmid (pMCK10) had *dhaB1* correctly oriented for transcription from the ADH1 promoter and contained a LEU2 marker.

Construction of *dhaB2* expression plasmid pMCK17

Plasmid pGADGH (Clontech, Palo Alto, CA) was digested with HindIII and the single-strand ends converted to EcoRI ends by ligation with HindIII-XmnI and EcoRI-XmnI adaptors (New England Biolabs, Beverly, MA). Selection for plasmids with correct EcoRI ends was achieved by ligation to a kanamycin resistance gene on an EcoRI fragment from plasmid pUC4K (Pharmacia Biotech, Uppsala), transformation into *E. coli* strain DH5a and selection on LB plates containing 25 mg/mL kanamycin. The resulting plasmid (pGAD/KAN2) was digested with SnaBI

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and EcoRI and a 1.8 kb fragment with the ADH1 promoter was isolated. Plasmid pGBT9 (Clontech, Palo Alto, CA) was digested with SnaBI and EcoRI, and the 1.5 kb ADH1/GAL4 fragment replaced by the 1.8 kb ADH1 promoter fragment isolated from pGAD/KAN2 by digestion with SnaBI and EcoRI. The resulting vector (pMCK11) is a replicating plasmid in yeast with an ADH1 promoter and terminator and a TRP1 marker. Plasmid pMCK11 was digested with EcoRI, dephosphorylated, and ligated to the *dhaB2* EcoRI fragment from pMP20. The resulting plasmid (pMCK17) had *dhaB2* correctly oriented for transcription from the ADH1 promoter and contained a TRP1 marker.

Construction of *dhaB3* expression plasmid pMCK30

Plasmid pGBT9 (Clontech) was digested with NaeI and PvuII and the 1 kb TRP1 gene removed from this vector. The TRP1 gene was replaced by a URA3 gene donated as a 1.7 kb AatII/NaeI fragment from plasmid pRS406 (Stratagene) to give the intermediary vector pMCK32. The truncated ADH1 promoter present on pMCK32 was removed on a 1.5 kb SnaBI/EcoRI fragment, and replaced with a full-length ADH1 promoter on a 1.8 kb SnaBI/EcoRI fragment from plasmid pGAD/KAN2 to yield the vector pMCK26. The unique EcoRI site on pMCK26 was used to insert an EcoRI fragment with *dhaB3* from plasmid pMP14 to yield pMCK30. The pMCK30 replicating expression plasmid has *dhaB3* orientated for expression from the ADH1 promoter, and has a URA3 marker.

Construction of *dhaT* expression plasmid pMCK35

Plasmid pGBT9 (Clontech) was digested with NaeI and PvuII and the 1 kb TRP1 gene removed from this vector. The TRP1 gene was replaced by a HIS3 gene donated as an XmnI/NaeI fragment from plasmid pRS403 (Stratagene) to give the intermediary vector pMCK33. The truncated ADH1 promoter present on pMCK33 was removed on a 1.5 kb SnaBI/EcoRI fragment, and replaced with a full-length ADH1 promoter on a 1.8 kb SnaBI/EcoRI fragment from plasmid pGAD/KAN2 to yield the vector pMCK31. The unique EcoRI site on pMCK31 was used to insert an EcoRI fragment with *dhaT* from plasmid pMP13 to yield pMCK35. The pMCK35 replicating expression plasmid has *dhaT* orientated for expression from the ADH1 promoter, and has a HIS3 marker.

Transformation of *S. cerevisiae* with *dha* expression plasmids

S. cerevisiae strain YPH500 (*ura3-52 lys2-801 ade2-101 trp1-D63 his3-D200 leu2-D1*) (Sikorski R. S. and Hieter P., *Genetics* 122, 19-27, (1989)) purchased from Stratagene (La Jolla, CA) was transformed with 1-2 mg of plasmid DNA using a Frozen-EZ Yeast Transformation Kit (Catalog #T2001) (Zymo Research, Orange, CA). Colonies were grown on Supplemented Minimal Medium (SMM - 0.67% yeast nitrogen base without amino acids, 2% glucose) for 3-4 d at 29 °C with one or more of the following additions: adenine sulfate (20 mg/L), uracil (20 mg/L),

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L-tryptophan (20 mg/L), L-histidine (20 mg/L), L-leucine (30 mg/L), L-lysine (30 mg/L). Colonies were streaked on selective plates and used to inoculate liquid media.

Screening of *S. cerevisiae* transformants for *dha* genes

Chromosomal DNA from URA⁺, HIS⁺, TRP⁺, LEU⁺ transformants was analyzed by PCR using primers specific for each gene (SEQ ID NOS:38-45). The presence of all four open reading frames was confirmed.

Expression of *dhaB* and *dhaT* activity in transformed *S. cerevisiae*

The presence of active glycerol dehydratase (*dhaB*) and 1,3-propanediol oxido-reductase (*dhaT*) was demonstrated using *in vitro* enzyme assays. Additionally, western blot analysis confirmed protein expression from all four open reading frames.

Strain YPH500, transformed with the group of plasmids pMCK10, pMCK17, pMCK30 and pMCK35, was grown on Supplemented Minimal Medium containing 0.67% yeast nitrogen base without amino acids 2% glucose 20 mg/L adenine sulfate, and 30 mg/L L-lysine. Cells were homogenized and extracts assayed for *dhaB* activity. A specific activity of 0.12 units per mg protein was obtained for glycerol dehydratase, and 0.024 units per mg protein for 1,3-propanediol oxido-reductase.

EXAMPLE 4

PRODUCTION OF 1,3-PROPANEDIOL FROM D-GLUCOSE
USING RECOMBINANT *Saccharomyces cerevisiae*

S. cerevisiae YPH500, harboring the groups of plasmids pMCK10, pMCK17, pMCK30 and pMCK35, was grown in a BiostatB fermenter (B Braun Biotech, Inc.) in 1.0 L of minimal medium initially containing 20 g/L glucose, 6.7 g/L yeast nitrogen base without amino acids, 40 mg/L adenine sulfate and 60 mg/L L-lysine·HCl. During the course of the growth, an additional equivalent of yeast nitrogen base, adenine and lysine was added. The fermenter was controlled at pH 5.5 with addition of 10% phosphoric acid and 2 M NaOH, 30 °C, and 40% dissolved oxygen tension through agitation control. After 38 h, the cells (OD₆₀₀ = 5.8 AU) were harvested by centrifugation and resuspended in base medium (6.7 g/L yeast nitrogen base without amino acids, 20 mg/L adenine sulfate, 30 mg/L L-lysine·HCl, and 50 mM potassium phosphate buffer, pH 7.0).

Reaction mixtures containing cells (OD₆₀₀ = 20 AU) in a total volume of 4 mL of base media supplemented with 0.5% glucose, 5 ug/mL coenzyme B₁₂ and 0, 10, 20, or 40 mM chloroquine were prepared, in the absence of light and oxygen (nitrogen sparging), in 10 mL crimp sealed serum bottles and incubated at 30 °C with shaking. After 30 h, aliquots were withdrawn and analyzed by HPLC. The results are shown in the Table 3.

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Table 3Production of 1,3-propanediol using recombinant *S. cerevisiae*

reaction	chloroquine (mM)	1,3-propanediol (mM)
1	0	0.2
2	10	0.2
3	20	0.3
4	40	0.7

EXAMPLE 5USE OF A *S. cerevisiae* DOUBLE TRANSFORMANT FOR PRODUCTION OF 1,3-PROPANEDIOL FROM D-GLUCOSE WHERE *dhaB* AND *dhaT* AREINTEGRATED INTO THE GENOME

Example 5 prophetically demonstrates the transformation of *S. cerevisiae* with *dhaB1*, *dhaB2*, *dhaB3*, and *dhaT* and the stable integration of the genes into the yeast genome for the production of 1,3-propanediol from glucose.

Construction of expression cassettes

Four expression cassettes (*dhaB1*, *dhaB2*, *dhaB3*, and *dhaT*) are constructed for glucose-induced and high-level constitutive expression of these genes in yeast, *Saccharomyces cerevisiae*. These cassettes consist of: (i) the phosphoglycerate kinase (PGK) promoter from *S. cerevisiae* strain S288C; (ii) one of the genes *dhaB1*, *dhaB2*, *dhaB3*, or *dhaT*; and (iii) the PGK terminator from *S. cerevisiae* strain S288C. The PCR-based technique of gene splicing by overlap extension (Horton et al., *BioTechniques*, 8:528-535, (1990)) is used to recombine DNA sequences to generate these cassettes with seamless joints for optimal expression of each gene. These cassettes are cloned individually into a suitable vector (pLITMUS 39) with restriction sites amenable to multi-cassette cloning in yeast expression plasmids.

Construction of yeast integration vectors

Vectors used to effect the integration of expression cassettes into the yeast genome are constructed. These vectors contain the following elements: (i) a polycloning region into which expression cassettes are subcloned; (ii) a unique marker used to select for stable yeast transformants; (iii) replication origin and selectable marker allowing gene manipulation in *E. coli* prior to transforming yeast. One integration vector contains the *URA3* auxotrophic marker (YIp352b), and a second integration vector contains the *LYS2* auxotrophic marker (pKP7).

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Construction of yeast expression plasmids

Expression cassettes for *dhaB1* and *dhaB2* are subcloned into the polycloning region of the Ylp352b (expression plasmid #1), and expression cassettes for *dhaB3* and *dhaT* are subcloned into the polycloning region of pKP7 (expression plasmid #2).

5 Transformation of yeast with expression plasmids

S. cerevisiae (*ura3*, *lys2*) is transformed with expression plasmid #1 using Frozen-EZ Yeast Transformation kit (Zymo Research, Orange, CA), and transformants selected on plates lacking uracil. Integration of expression cassettes for *dhaB1* and *dhaB2* is confirmed by PCR analysis of chromosomal DNA. Selected transformants are re-transformed with expression
10 plasmid #2 using Frozen-EZ Yeast Transformation kit, and double transformants selected on plates lacking lysine. Integration of expression cassettes for *dhaB3* and *dhaT* is confirmed by PCR analysis of chromosomal DNA. The presence of all four expression cassettes (*dhaB1*, *dhaB2*, *dhaB3*, *dhaT*) in double transformants is confirmed by PCR analysis of chromosomal DNA.

15 Protein production from double-transformed yeast

Production of proteins encoded by *dhaB1*, *dhaB2*, *dhaB3* and *dhaT* from double-transformed yeast is confirmed by Western blot analysis.

Enzyme activity from double-transformed yeast

Active glycerol dehydratase and active 1,3-propanediol dehydrogenase from double-
20 transformed yeast is confirmed by enzyme assay as described in General Methods above.

Production of 1,3-propanediol from double-transformed yeast

Production of 1,3-propanediol from glucose in double-transformed yeast is demonstrated essentially as described in Example 4.

EXAMPLE 625 CONSTRUCTION OF PLASMIDS CONTAINING DAR1/GPP2OR *dhaT/dhaB1-3* AND TRANSFORMATION INTO *KLEBSIELLA* SPECIES

K. pneumoniae (ATCC 25955), *K. pneumoniae* (ECL2106), and *K. oxytoca* (ATCC 8724) are naturally resistant to ampicillin (up to 150 ug/mL) and kanamycin (up to 50 ug/mL), but sensitive to tetracycline (10 ug/mL) and chloramphenicol (25 ug/mL). Consequently, replicating
30 plasmids which encode resistance to these latter two antibiotics are potentially useful as cloning vectors for these *Klebsiella* strains. The wild-type *K. pneumoniae* (ATCC 25955), the glucose-derepressed *K. pneumoniae* (ECL2106), and *K. oxytoca* (ATCC 8724) were successfully transformed to tetracycline resistance by electroporation with the moderate-copy-number plasmid, pBR322 (New England Biolabs, Beverly, MA). This was accomplished by the following
35 procedure: Ten mL of an overnight culture was inoculated into 1 L LB (1% (w/v) Bacto-tryptone (Difco, Detroit, MI), 0.5% (w/v) Bacto-yeast extract (Difco) and 0.5% (w/v) NaCl (Sigma, St. Louis,

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MO) and the culture was incubated at 37 °C to an OD₆₀₀ of 0.5-0.7. The cells were chilled on ice, harvested by centrifugation at 4000 x g for 15 min, and resuspended in 1 L ice-cold sterile 10% glycerol. The cells were repeatedly harvested by centrifugation and progressively resuspended in 500 mL, 20 mL and, finally, 2 mL ice-cold sterile 10% glycerol. For electroporation, 40 uL of cells were mixed with 1-2 uL DNA in a chilled 0.2 cm cuvette and were pulsed at 200 Ω, 2.5 kV for 4-5 msec using a BioRad Gene Pulser (BioRad, Richmond, CA). One mL of SOC medium (2% (w/v) Bacto-tryptone (Difco), 0.5% (w/v) Bacto-yeast extract (Difco), 10 mM NaCl, 10 mM MgCl₂, 10 mM MgSO₄, 2.5 mM KCl and 20 mM glucose) was added to the cells and, after the suspension was transferred to a 17 x 100 mm sterile polypropylene tube, the culture was incubated for 1 hr at 37 °C, 225 rpm. Aliquots were plated on selective medium, as indicated. Analyses of the plasmid DNA from independent tetracycline-resistant transformants showed the restriction endonuclease digestion patterns typical of pBR322, indicating that the vector was stably maintained after overnight culture at 37 °C in LB containing tetracycline (10 ug/mL). Thus, this vector, and derivatives such as pBR329 (ATCC 37264) which encodes resistance to ampicillin, tetracycline and chloramphenicol, may be used to introduce the *DAR1/GPP2* and *dhaT/dhaB1-3* expression cassettes into *K. pneumoniae* and *K. oxytoca*.

The *DAR1* and *GPP2* genes may be obtained by PCR-mediated amplification from the *Saccharomyces cerevisiae* genome, based on their known DNA sequence. The genes are then transformed into *K. pneumoniae* or *K. oxytoca* under the control of one or more promoters that may be used to direct their expression in media containing glucose. For convenience, the genes were obtained on a 2.4 kb DNA fragment obtained by digestion of plasmid pAH44 with the *PvuII* restriction endonuclease, whereby the genes are already arranged in an expression cassette under the control of the *E. coli lac* promoter. This DNA fragment was ligated to *PvuII*-digested pBR329, producing the insertional inactivation of its chloramphenicol resistance gene. The ligated DNA was used to transform *E. coli* DH5α (Gibco, Gaithersburg, MD). Transformants were selected by their resistance to tetracycline (10 ug/mL) and were screened for their sensitivity to chloramphenicol (25 ug/mL). Analysis of the plasmid DNA from tetracycline-resistant, chloramphenicol-sensitive transformants confirmed the presence of the expected plasmids, in which the *P_{lac}-dar1-gpp2* expression cassette was subcloned in either orientation into the pBR329 *PvuII* site. These plasmids, designated pJSP1A (clockwise orientation) and pJSP1B (counterclockwise orientation), were separately transformed by electroporation into *K. pneumonia* (ATCC 25955), *K. pneumonia* (ECL2106) and *K. oxytoca* (ATCC 8724) as described. Transformants were selected by their resistance to tetracycline (10 ug/mL) and were screened for their sensitivity to chloramphenicol (25 ug/mL). Restriction analysis of the plasmids isolated from independent transformants showed only the expected digestion patterns, and confirmed that they were stably maintained at 37 °C with antibiotic selection. The expression of the *DAR1* and *GPP2* genes may be enhanced by the addition of IPTG (0.2-2.0 mM) to the growth medium.

The four *K. pneumoniae* *dhaB*(1-3) and *dhaT* genes may be obtained by PCR-mediated amplification from the *K. pneumoniae* genome, based on their known DNA sequence. These genes are then transformed into *K. pneumoniae* under the control of one or more promoters that may be used to direct their expression in media containing glucose. For convenience, the genes were obtained on an approximately 4.0 kb DNA fragment obtained by digestion of plasmid pAH24 with the *KpnI/SacI* restriction endonucleases, whereby the genes are already arranged in an expression cassette under the control of the *E. coli lac* promoter. This DNA fragment was ligated to similarly digested pBC-KS+ (Stratagene, LaJolla, CA) and used to transform *E. coli* DH5 α . Transformants were selected by their resistance to chloramphenicol (25 ug/mL) and were screened for a white colony phenotype on LB agar containing X-gal. Restriction analysis of the plasmid DNA from chloramphenicol-resistant transformants demonstrating the white colony phenotype confirmed the presence of the expected plasmid, designated pJSP2, in which the *dhaT-dhaB*(1-3) genes were subcloned under the control of the *E. coli lac* promoter.

To enhance the conversion of glucose to 1,3-propanediol, this plasmid was separately transformed by electroporation into *K. pneumoniae* (ATCC 25955) (pJSP1A), *K. pneumoniae* (ECL2106) (pJSP1A) and *K. oxytoca* (ATCC 8724) (pJSP1A) already containing the *P_{lac}-dar1-gpp2* expression cassette. Cotransformants were selected by their resistance to both tetracycline (10 ug/mL) and chloramphenicol (25 ug/mL). Restriction analysis of the plasmids isolated from independent cotransformants showed the digestion patterns expected for both pJSP1A and pJSP2. The expression of the *DAR1*, *GPP2*, *dhaB*(1-3), and *dhaT* genes may be enhanced by the addition of IPTG (0.2-2.0 mM) to the medium.

EXAMPLE 7

Production of 1,3 propanediol from glucose by *K. pneumoniae*

Klebsiella pneumoniae strains ECL 2106 and 2106-47, both transformed with pJSP1A, and ATCC 25955, transformed with pJSP1A and pJSP2, were grown in a 5 L Applikon fermenter under various conditions (see Table 4) for the production of 1,3-propanediol from glucose. Strain 2104-47 is a fluoroacetate-tolerant derivative of ECL 2106 which was obtained from a fluoroacetate/lactate selection plate as described in Bauer et al., *Appl. Environ. Microbiol.* 56, 1296 (1990). In each case, the medium used contained 50-100 mM potassium phosphate buffer, pH 7.5, 40 mM (NH₄)₂SO₄, 0.1% (w/v) yeast extract, 10 μ M CoCl₂, 6.5 μ M CuCl₂, 100 μ M FeCl₃, 18 μ M FeSO₄, 5 μ M H₃BO₃, 50 μ M MnCl₂, 0.1 μ M Na₂MoO₄, 25 μ M ZnCl₂, 0.82 mM MgSO₄, 0.9 mM CaCl₂, and 10-20 g/L glucose. Additional glucose was fed, with residual glucose maintained in excess. Temperature was controlled at 37 °C and pH controlled at 7.5 with 5N KOH or NaOH. Appropriate antibiotics were included for plasmid maintenance; IPTG (isopropyl-b-D-thiogalactopyranoside) was added at the indicated concentrations as well. For anaerobic fermentations, 0.1 vvm nitrogen was sparged through the reactor; when the dO

setpoint was 5%, 1 vvm air was sparged through the reactor and the medium was supplemented with vitamin B12. Final concentrations and overall yields (g/g) are shown in Table 4.

Table 4

Production of 1,3 propanediol from glucose by *K. pneumoniae*

Organism	dO	IPTG, mM	vitamin B12, mg/L	Titer, g/L	Yield, g/g
25955[pJSP1A/pJS P2]	0	0.5	0	8.1	16%
25955[pJSP1A/pJS P2]	5%	0.2	0.5	5.2	4%
2106[pJSP1A]	0	0	0	4.9	17%
2106[pJSP1A]	5%	0	5	6.5	12%
2106-47[pJSP1A]	5%	0.2	0.5	10.9	12%

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EXAMPLE 8

Conversion of carbon substrates to 1,3-propanediol by recombinant

K. pneumoniae containing *dar1*, *gpp2*, *dhaB*, and *dhaT*

A. Conversion of D-fructose to 1,3-propanediol by various *K. pneumoniae* recombinant strains:

10 Single colonies of *K. pneumoniae* (ATCC 25955 pJSP1A), *K. pneumoniae* (ATCC 25955 pJSP1A/pJSP2), *K. pneumoniae* (ATCC 2106 pJSP1A), and *K. pneumoniae* (ATCC 2106 pJSP1A/pJSP2) were transferred from agar plates and in separate culture tubes were subcultured overnight in Luria-Bertani (LB) broth containing the appropriate antibiotic agent(s). A 50-mL flask containing 45 mL of a steri-filtered minimal medium defined as LLMM/F which
15 contains per liter: 10 g fructose; 1 g yeast extract; 50 mmoles potassium phosphate, pH 7.5; 40 mmoles (NH₄)₂SO₄; 0.09 mmoles calcium chloride; 2.38 mg CoCl₂·6H₂O; 0.88 mg CuCl₂·2H₂O; 27 mg FeCl₃·6H₂O; 5 mg FeSO₄·7H₂O; 0.31 mg H₃BO₃; 10 mg MnCl₂·4H₂O; 0.023 mg Na₂MoO₄·2H₂O; 3.4 mg ZnCl₂; 0.2 g MgSO₄·7H₂O. Tetracycline at 10 ug/mL was added to medium for reactions using either of the single plasmid recombinants; 10 ug/mL
20 tetracycline and 25 ug/mL chloramphenicol for reactions using either of the double plasmid recombinants. The medium was thoroughly sparged with nitrogen prior to inoculation with 2 mL of the subculture. IPTG (I) at final concentration of 0.5 mM was added to some flasks. The flasks were capped, then incubated at 37 °C, 100 rpm in a New Brunswick Series 25 incubator/shaker. Reactions were run for at least 24 hours or until most of the carbon substrate was converted into
25 products. Samples were analyzed by HPLC. Table 5 describes the yields of 1,3-propanediol (3G) produced from fructose by the various *Klebsiella* recombinants.

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Table 5

Production of 1,3-propanediol from D-fructose using recombinant *Klebsiella*

Klebsiella Strain	Medium	Conversion	[3G] (g/L)	Yield Carbon (%)
2106 pBR329	LLMM/F	100	0	0
2106 pJSP1A	LLMM/F	50	0.66	15.5
2106 pJSP1A	LLMM/F + I	100	0.11	1.4
2106 pJSP1A/pJSP2	LLMM/F	58	0.26	5
25955 pBR329	LLMM/F	100	0	0
25955 pJSP1A	LLMM/F	100	0.3	4
25955 pJSP1A	LLMM/F + I	100	0.15	2
25955 pJSP1A/pJSP2	LLMM/F	100	0.9	11
25955	LLMM/F + I	62	1.0	20

B. Conversion of various carbon substrates to 1,3-propanediol by *K. pneumoniae* (ATCC 25955 pJSP1A/pJSP2):

An aliquot (0.1 mL) of frozen stock cultures of *K. pneumoniae* (ATCC 25955 pJSP1A/pJSP2) was transferred to 50 mL Seed medium in a 250 mL baffled flask. The Seed medium contained per liter: 0.1 molar NaK/PO₄ buffer, pH 7.0; 3 g (NH₄)₂SO₄; 5 g glucose, 0.15 g MgSO₄·7H₂O, 10 mL 100X Trace Element solution, 25 mg chloramphenicol, 10 mg tetracycline, and 1 g yeast extract. The 100X Trace Element contained per liter: 10 g citric acid, 1.5 g CaCl₂·2H₂O, 2.8 g FeSO₄·7H₂O, 0.39 g ZnSO₄·7H₂O, 0.38 g CuSO₄·5H₂O, 0.2 g CoCl₂·6H₂O, and 0.3 g MnCl₂·4H₂O. The resulting solution was titrated to pH 7.0 with either KOH or H₂SO₄. The glucose, trace elements, antibiotics and yeast extracts were sterilized separately. The seed inoculum was grown overnight at 35 °C and 250 rpm.

The reaction design was semi-aerobic. The system consisted of 130 mL Reaction medium in 125 mL sealed flasks that were left partially open with aluminum foil strip. The Reaction Medium contained per liter: 3 g (NH₄)₂SO₄; 20 g carbon substrate; 0.15 molar NaK/PO₄ buffer, pH 7.5; 1 g yeast extract; 0.15 g MgSO₄·7H₂O; 0.5 mmoles IPTG; 10 mL 100X Trace Element solution; 25 mg chloramphenicol; and 10 mg tetracycline. The resulting solution was titrated to pH 7.5 with KOH or H₂SO₄. The carbon sources were: D-glucose (Glc); D-fructose (Frc); D-lactose (Lac); D-sucrose (Suc); D-maltose (Mal); and D-mannitol (Man). A few glass beads were included in the medium to improve mixing. The reactions were initiated by addition of seed inoculum so that the optical density of the cell suspension started at 0.1 AU as

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measured at 600 nm. The flasks were incubated at 35 °C: 250 rpm. 3G production was measured by HPLC after 24 hr. Table 6 describes the yields of 1,3-propanediol produced from the various carbon substrates.

5

Table 6
Production of 1,3-propanediol from various carbon substrates
using recombinant *Klebsiella* 25955 pJSP1A/pJSP2

Carbon Substrate	1,3-Propanediol (g/L)		
	Expt. 1	Expt. 2	Expt 3
Glc	0.89	1	1.6
Frc	0.19	0.23	0.24
Lac	0.15	0.58	0.56
Suc	0.88	0.62	
Mal	0.05	0.03	0.02
Man	0.03	0.05	0.04

10

EXAMPLE 9

IMPROVEMENT OF 1,3-PROPANEDIOL PRODUCTION USING *dhaBX* GENE

Example 9 demonstrates the improved production of 1,3-propanediol in *E.coli* when a gene encoding a protein X is introduced.

Construction of expression vector pTacIQ

15

The *E. coli* expression vector, pTacIQ containing the lacIq gene (Farabaugh, P.J. 1978, Nature 274 (5673) 765-769) and tac promoter (Amann et al, 1983, Gene 25, 167-178) was inserted into the restriction endonuclease site EcoRI of pBR322 (Sutcliffe, 1979, Cold Spring Harb. Symp. Quant. Biol. 43, 77-90). A multiple cloning site and terminator sequence (SEQ ID NO:50) replaces the pBR322 sequence from EcoRI to SphI.

20

Subcloning the glycerol dehydratase genes (*dhaB1*, 2,3, X)

The region containing the entire coding region for *Klebsiella dhaB1*, *dhaB2*, *dhaB3* and *dhaBX* of the *dhaB* operon from pHK28-26 was cloning into pBluescriptIIKS+(Stratagene) using the restriction enzymes KpnI and EcoRI to create the plasmid pM7.

25

The open reading frame for *dhaB3* gene was amplified from pHK 28-26 by PCR using primers (SEQ ID NO:51 and SEQ ID NO:52) incorporating an EcoRI site at the 5' end and a XbaI site at the 3' end. The product was subcloned into pLitmus29(NEB) to generate the plasmid pDHAB3 containing *dhaB3*.

30

The *dhaBX* gene was removed by digesting plasmid pM7 with Apal and XbaI, purifying the 5.9 kb fragment and ligating it with the 325-bp Apal-XbaI fragment from plasmid pDHAB3 to create pM11 containing *dhaB1*, *dhaB2* and *dhaB3*.

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The open reading frame for the *dhaB1* gene was amplified from pHK28-26 by PCR using primers (SEQ ID NO:53 and SEQ ID NO:54) incorporating HindIII site and a consensus ribosome binding site at the 5' end and a XbaI site at the 3' end. The product was subcloned into pLitmus28(NEB) to generate the plasmids pDT1 containing *dhaB1*.

5 A NotI-XbaI fragment from pM11 containing part of the *dhaB1* gene, the *dhaB2* gene and the *dhaB3* gene with inserted into pDT1 to create the *dhaB* expression plasmid, pDT2. The HindIII-XbaI fragment containing the *dhaB(1,2,3)* genes from pDT2 was inserted into pTaclQ to create pDT3.

Subcloning the TMG dehydrogenase gene (*dhaT*)

10 The KpnI-SacI fragment of pHK28-26, containing the TMG dehydrogenase (*dhaT*) gene, was subcloned into pBluescriptII KS+ creating plasmid pAH1. The *dhaT* gene was cloned by PCR from pAH1 as template DNA and synthetic primers (SEQ ID NO:55 with SEQ ID NO:56) incorporating an XbaI site at the 5' end and a BamHI site at the 3' end. The product was subcloned into pCR-Script(Stratagene) at the SrfI site to generate the plasmids pAH4 and pAH5
15 containing *dhaT*. The pAH4 contains the *dhaT* gene in the right orientation for expression from the lac promoter in pCR-Script and pAH5 contains *dhaT* gene in the opposite orientation. The XbaI-BamHI fragment from pAH4 containing the *dhaT* gene was inserted into pTaclQ to generate plasmid, pAH8. The HindII-BamHI fragment from pAH8 containing the RBS and *dhaT* gene was inserted into pBluescriptIIKS+ to create pAH11.

Construction of an expression cassette for *dhaT* and *dhaB(1,2,3)*

20 An expression cassette for *dhaT* and *dhaB(1,2,3)* was assembled from the individual *dhaB(1,2,3)* and *dhaT* subclones described previously using standard molecular biology methods. A SpeI-SacI fragment containing the *dhaB(1,2,3)* genes from pDT3 was inserted into pAH11 at the SpeI-SacI sites to create pAH24. A Sall-XbaI linker (SEQ ID NO 57 and SEQ ID NO 58) was
25 inserted into pAH5 which was digested with the restriction enzymes Sall-XbaI to create pDT16. The linker destroys the XbaI site. The 1 kb Sall-MluI fragment from pDT16 was then inserted into pAH24 replacing the existing Sall-MluI fragment to create pDT18.

Plasmid for the over-expression of *dhaT* and *dhaB(1, 2, 3, X)* in *E. coli*

30 The 4.4 kb NotI-XbaI fragment containing part of the *dhaB1* gene, *dhaB2*, *dhaB3* and *dhaBX* from plasmid pM7 was purified and ligated with the 4.1 Kb NotI-XbaI fragment from plasmid pDT18 (restoring *dhaB1*) to create pM33 containing the *dhaB1*, *dhaB2*, *dhaB3* and *dhaBX*.

E. coli strain

35 *E. coli* DH5a was obtained from BRL (Difco). This strain was transformed with the plasmids pM7, pM11, pM33 or pDT18 and selected on LA plates containing 100 µg/ml carbenicillin.

Production of 1,3-propanediol

E. coli DH5a, containing plasmid pM7, pM11, pM33 or pDT18 was grown on LA plates plus 100 ug/ml carbenicillin overnight at 37°C. One colony from each was used to inoculate 25 ml of media (0.2 M KH₂PO₄, citric acid 2.0 g/L, MgSO₄·7H₂O 2.0 g/L, H₂SO₄ (98%) 1.2 ml/L, Ferric ammonium citrate 0.3 g/L, CaCl₂·2H₂O 0.2 gram, yeast extract 5 g/L, glucose 10 g/L, glycerol 30 g/L,) plus Vitamine B12 0.005 g/L, 0.2 mM IPTG, 200 ug/ml carbenicillin and 5 ml modified Balch's trace-element solution (the composition of which can be found in Methods for General and Molecular Bacteriology (P. Gerhardt et al., eds, p 158, American Society for Microbiology, Washington, DC 1994), final pH 6.8 (NH₄OH), then filter-sterilized in 250 ml erlenmeyers flasks. The shake flasks were incubated at 37°C with shaking (300 rpm) for several days, during which they were sampled for HPLC analysis by standard procedures. Final yields are shown in Table 4.

Overall, as shown in Table 7, the results indicate that the expression of *dhaBX* in plasmids expressing *dhaB*(1,2,3) or *dhaT-dhaB*(1,2,3) greatly enhances the production of 1,3-propanediol.

TABLE 7

Effect of *dhaBX* expression on the production of 1,3-propanediol by *E. coli*

Strain	Time (days)	1,3-propanediol (mg/L)*
DH5a/pM7 (<i>dhaB</i> 1,2,3,X)	1	1500
	2	2700
DH5a/pM11 (<i>dhaB</i> 1,2,3)	1	< 200 µg
	2	< 200 µg
DH5a/pM33 (<i>dhaT-dhaB</i> 1,2,3,X)	2	1200
DH5a/pDT18 (<i>dhaT-dhaB</i> 1,2,3)	2	88

* Expressed as an average from several experiments.

Primers:

SEQ ID NO: 50- MCS-TERMINATOR:

5 AGCTTAGGAGTCTAGAATATTGAGCTCGAATTCCTGGGCATGCGGTACCGGATCCAGAAAA
AAGCCCGCACCTGACAGTGCGGGCTTTTTTTTTT 3'

SEQ ID NO: 51 -*dhaB*3-5' end. EcoRI

GGAATTCAGATCTCAGCAATGAGCGAGAAAACCATGC

SEQ ID NO 52: *dhaB*3-3' end XbaI

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GCTCTAGATTAGCTTCCTTTACGCAGC

SEQ ID NO 53: *dhaB1* 5' end-HindIII-SD

5' GGCCAAGCTTAAGGAGGTTAATTAAATGAAAAG 3'

5

SEQ ID NO 54: *dhaB1* 3' end-XbaI

5' GCTCTAGATTATTCAATGGTGTCTGGG 3'

SEQ ID NO 55: *dhaT* 5' end-XbaI

10 5' GCGCCGTCTAGAATTATGAGCTATCGTATGTTTGATTATCTG 3'

SEQ ID NO 56: *dhaT* 3' end-BamHI

5' TCTGATACGGGATCCTCAGAATGCCTGGCGGAAAAT 3'

15 SEQ ID NO 57: pUSH Linker1:

5' TCGACGAATTCAGGAGGA 3'

SEQ ID NO 58: pUSH Linker2:

5' CTAGTCCTCCTGAATTCG 3'

20

EXAMPLE 10

Reactivation of the Glycerol Dehydratase Activity

Example 10 demonstrates the *in vivo* reactivation of the glycerol dehydratase activity in microorganisms containing at least one gene encoding protein X.

25 Plasmids pM7 and pM11 were constructed as described in Example 9 and transformed into *E. coli* DH5 α cells. The transformed cells were cultured and assayed for the production of 1,3-propanediol according to the method of Honda et al. (1980, In Situ Reactivation of Glycerol-Inactivated Coenzyme B₁₂-Dependent Enzymes, Glycerol Dehydratase and Diol Dehydratase. Journal of Bacteriology 143:1458-1465).

30 Materials and methods

Toluenization of Cells

The cells were grown to mid-log phase and were harvested by centrifugation at room temperature early in growth, i.e. $0.2 > OD_{600} < 0.8$. The harvested cells were washed 2x in 50mM KPO₄ pH8.0 at room temperature. The cells were resuspended to OD₆₀₀ 20-30 in 50mM KPO₄ pH8.0. The absolute OD is not critical. A lower cell mass is resuspend in less volume. If
35 coenzyme B12 is added at this point, the remainder of the steps are performed in the dark.

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Toluene is added to 1% final volume of cell suspension and the suspension is shaken vigorously for 5 minutes at room temperature. The suspension is centrifuged to pellet the cells. The cells are washed 2x in 50mM KPO₄ pH8.0 at room temperature (25mls each). The cell pellet is resuspended in the same volume as was used prior to toluene addition and transfer to fresh tubes. The OD₆₀₀ for the toluenized cells was measured and recorded and stored at 4 degrees C..

Whole Cell Glycerol Dehydratase Assay

The toluene treated cells were assayed at 37 degrees C for the presence of dehydratase activity. Three sets of reactions were carried out as shown below: no ATP, ATP added at 0 time, and ATP added at 10 minutes.

10	No ATP:	100ul	2M Glycerol
		100ul	150uM CoB ₁₂
		700ul	Buffer (0.03M KPO ₄ / 0.5M KCl, pH8.0)
15	T=0 minute ATP	100ul	2M Glycerol
		100ul	150uM CoB ₁₂
		600ul	Buffer (0.03M KPO ₄ / 0.5M KCl, pH8.0)
		100ul	30mM ATP/ 30mM MnCl ₂
20	T=10 minute ATP	100ul	2M Glycerol
		100ul	150uM CoB ₁₂
		700ul	Buffer (0.03M KPO ₄ / 0.5M KCl, pH8.0)

Controls were prepared for each of the above conditions by adding 100uls buffer instead of CoB₁₂. The tubes were mixed. 50uls MBTH (3-Methyl-2-Benzo-Thiazolinone Hydrazone) (6 mg/ml in 375mM Glycine / HCl pH2.7) was added to each of these tubes and continue incubation in ice water. The reaction tubes were placed in a 37 degree C water bath for a few minutes to equilibrate to 37 degree C. A tube containing enough toluenized cells for all assay tubes was placed into the 37 degree C water bath for a few minutes to equilibrate to 37 degree C. A tube containing 2.5 fold diluted (in assay buffer) 30mM ATP/ 30mM MnCl₂ (12mM each) was placed into the 37 degree C water bath for a few minutes to equilibrate to 37 degree C. A 100ul cell suspension was added to all tubes and samples were taken at 0,1,2,3,4,5,10,15,20 and 30 minutes. At every timepoint, 100uls of reaction was withdrawn and immediately added to 50uls ice cold MBTH, vortexed, and placed in an ice water bath. At T=10 minutes, a sample was withdrawn and added to MBTH, then 100uls of the 2.5 fold diluted ATP/Mn was added as fast as is possible. When all samples were collected, the sample tube rack was added to a boiling water bath and boiled for three minutes. The tubes were chilled in an ice water bath for 30 seconds.

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500ul of freshly prepared 3.3 mg/ml $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, was added to the tubes and the tubes vortexed. The tubes were incubated at room temperature for 30 minutes, diluted 10x in H_2O , and then centrifuged to collect the cells and particulates. The absorbance was measured at 670nm and the cells were diluted to keep OD under 1.0.

Example of Calculation of Activity

The observed OD670 was multiplied by the dilution factor to determine absorbance. The blank absorbance was subtracted for that reaction series and the T0 A670nm was subtracted. The absolute A670nm was divided by 53.4 (mM extinction coefficient for 3OH-propionaldehyde) and the mM concentration was multiplied by any dilution of reaction during timecourse. Because 1 ml reaction was used, the concentration (umoles/ml) of 3OH-propionaldehyde was divided by the mgs dry weight used in the assay (calculated via OD600 and $1\text{OD } 600 = 0.436\text{ mgs dry weight}$) to get umoles aldehyde per mg dry weight cells.

Results

As shown in Figure 6, whole E.coli cells were assayed for reactivation of glycerol dehydratase in the absence and presence of added ATP and Mn^{++} . The results indicate that cells containing a plasmid carrying dhaB 1, 2 and 3 as well as protein X have the ability to reactivate catalytically inactivated glycerol dehydrogenase. Cells containing protein 1, protein 2 and protein 3 have increased ability to reactivate the catalytically inactivated glycerol dehydratase.

As shown in Figure 7, whole E.coli cells were assayed for reactivation of glycerol-inactivated glycerol dehydratase in the absence and in the presence of added ATP and Mn^{++} . The results show that cells containing dhaB subunits 1, 2 and 3 and X have the ability to reactivate catalytically inactivated glycerol dehydratase. Cell lacking the protein X gene do not have the ability to reactivate the catalytically inactivated glycerol dehydratase.

Figures 9 and 10 illustrate that host cells containing plasmid pHK 28-26 (Figure 1), when cultured under conditions suitable for the production of 1,3-propanediol, produced more 1,3-propanediol than host cells transformed with pDT24 and cultured under conditions suitable for the production of 1,3-propanediol. Plasmid pDT24 is a derivative of pDT18 (described in Example 9) and contains dhaT, dhaB 1, 2, 3 and protein X, but lacks proteins 1, 2 and 3.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: MARIA DIAZ-TORRES
NIGEL DUNN-COLEMAN
MATTHEW CHASE
- (ii) TITLE OF INVENTION: METHOD FOR THE
RECOMBINANT PRODUCTION OF 1,3 PROPANEDIOL
- (iii) NUMBER OF SEQUENCES: 49
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: GENENCOR INTERNATIONAL, INC.
 - (B) STREET: 4 CAMBRIDGE PLACE
1870 SOUTH WINTON ROAD
 - (C) CITY: ROCHESTER
 - (D) STATE: NEW YORK
 - (E) COUNTRY: U.S.A.
 - (F) POSTAL CODE (ZIP): 14618
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: 3.50 INCH DISKETTE
 - (B) COMPUTER: IBM PC COMPATIBLE
 - (C) OPERATING SYSTEM: MICROSOFT WINDOWS 3.1
 - (D) SOFTWARE: MICROSOFT WORD 2.0C
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE: 11/13/97
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 60/030,601
 - (B) FILING DATE: 11/13/96
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: GLAISTER, DEBRA
 - (B) REGISTRATION NO.: 33,888
 - (C) REFERENCE/DOCKET NUMBER: GC 369-2
 - (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 650-864-7620
 - (B) TELEFAX: 650-845-6504

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1668 base pairs

-- 53 --

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: DHAB1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GACATGATCG ACCGATTTAT CGCCGATTAC GCGATCAACG TTGAGCGCAC AGAGCAGGCA	240
ATGCGCCTGG AGGCGGTGGA AATAGCCCGT ATGCTGGTGG ATATTCACGT CAGCCGGGAG	300
GAGATCATTG CCATCACTAC CGCCATCACG CCGGCCAAAG CGGTCGAGGT GATGGCGCAG	360
ATGAACGTGG TGGAGATGAT GATGGCGCTG CAGAAGATGC GTGCCCCGCCG GACCCCTCC	420
AACCAAGTGC ACGTCACCAA TCTCAAAGAT AATCCGGTGC AGATTGCCGC TGACGCCGCC	480
GAGGCCGGA TCCGCGGCTT CTCAGAACAG GAGACCACGG TCGGTATCGC GCGCTACGCG	540
CCGTTTAACG CCCTGGCGCT GTTGGTCGGT TCGCAGTGCG GCCGCCCCGG CGTGTTGACG	600
CAGTGCTCGG TGGAAGAGGC CACCGAGCTG GAGCTGGGCA TCGGTGGCTT AACCAGCTAC	660
GCCGAGACGG TGTCGGTCTA CGGCACCGAA GCGGTATTTA CCGACGGCGA TGATACGCCG	720
TGGTCAAAGG CGTTCCTCGC CTCGGCCTAC GCCTCCCGCG GGTGAAAAT GCGCTACACC	780
TCCGGCACCG GATCCGAAGC GCTGATGGGC TATTCGGAGA GCAAGTCGAT GCTCTACCTC	840
GAATCGCGCT GCATCTTCAT TACTAAAGGC GCCGGGGTTC AGGGACTGCA AAACGGCGCG	900
GTGAGCTGTA TCGGCATGAC CGGCGCTGTG CCGTCGGGCA TTCGGGCGGT GCTGGCGGAA	960
AACCTGATCG CCTCTATGCT CGACCTCGAA GTGGCGTCCG CCAACGACCA GACTTTCTCC	1020
CACTCGGATA TTCGCCGCAC CGCGCGCACC CTGATGCAGA TGCTGCCGGG CACCGACTTT	1080
ATTTTCTCCG GCTACAGCGC GGTGCCGAAC TACGACAACA GTTTCGCCGG CTCGAACTTC	1140
GATGCGGAAG ATTTTGATGA TTACAACATC CTGCAGCGTG ACCTGATGGT TGACGGCGGC	1200
CTGCGTCCGG TGACCGAGGC GGAAACCATT GCCATTCGCC AGAAAGCGGC GCGGGCGATC	1260

-- 54 --

CAGGCGGTTT TCCGCGAGCT GGGGCTGCCG CCAATCGCCG ACGAGGAGGT GGAGGCCGCC 1320
ACCTACGCGC ACGGCAGCAA CGAGATGCCG CCGCGTAACG TGGTGGAGGA TCTGAGTGCG 1380
GTGGAAGAGA TGATGAAGCG CAACATCACC GGCCTCGATA TTGTCGGCGC GCTGAGCCGC 1440
AGCGGCTTTG AGGATATCGC CAGCAATATT CTCAATATGC TGCGCCAGCG GGTACCCGGC 1500
GATTACCTGC AGACCTCGGC CATTCTCGAT CGGCAGTTCG AGGTGGTGAG TCGGGTCAAC 1560
GACATCAATG ACTATCAGGG GCCGGGCACC GGCTATCGCA TCTCTGCCGA ACGCTGGGCG 1620
GAGATCAAAA ATATTCCGGG CGTGGTTCAG CCCGACACCA TTGAATAA 1668

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 585 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: DHAB2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTGCAACAGA CAACCCAAAT TCAGCCCTCT TTTACCCTGA AAACCCGCGA GGGCGGGGTA 60
GCTTCTGCCG ATGAACGCGC CGATGAAGTG GTGATCGGCG TCGGCCCTGC CTTGATAAAA 120
CACCAGCATC AACTCTGAT CGATATGCCC CATGGCGCGA TCCTCAAAGA GCTGATTGCC 180
GGGGTGAAG AAGAGGGGCT TCACGCCCGG GTGGTGCGCA TTCTGCGCAC GTCCGACGTC 240
TCCTTTATGG CCTGGGATGC GGCCAACCTG AGCGGCTCGG GGATCGGCAT CGGTATCCAG 300
TCGAAGGGGA CCACGGTCAT CCATCAGCGC GATCTGCTGC CGCTCAGCAA CCTGGAGCTG 360
TTCTCCCAGG CGCCGCTGCT GACGCTGGAG ACCTACCGGC AGATTGGCAA AAACGCTGCG 420
CGCTATGCGC GCAAAGAGTC ACCTTCGCCG GTGCCGGTGG TGAACGATCA GATGGTGCGG 480
CCGAAATTTA TGGCCAAAGC CGCGCTATTT CATATCAAAG AGACCAAACA TGTGGTGCAG 540
GACGCCGAGC CCGTCACCCT GCACATCGAC TTAGTAAGGG AGTGA 585

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 426 base pairs
- (B) TYPE: nucleic acid

- 55 -

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: DHAB3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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ATGAGCGAGA AAACCATGCG CGTGCAGGAT TATCCGTTAG CCACCCGCTG CCCGGAGCAT    60
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GCCGAGCAGA TGCAGCGCCA TGCGGTGGCG CGCAATTTCC GCCGCGCGGC GGAGCTTATC    240
GCCATTCTTG ACGAGCGCAT TCTGGCTATC TATAACGCGC TGCGCCCGTT CCGCTCCTCG    300
CAGGCGGAGC TGCTGGCGAT CGCCGACGAG CTGGAGCACA CCTGGCATGC GACAGTGAAT    360
GCCGCCTTTG TCCGGGAGTC GCGGGAAGTG TATCAGCAGC GGCATAAGCT GCGTAAAGGA    420
AGCTAA                                         426

```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1164 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: DHAT

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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ATGAGCTATC GTATGTTTGA TTATCTGGTG CCAAACGTTA ACTTTTTTGG CCCCAACGCC    60
ATTTCCGTAG TCGGCGAACG CTGCCAGCTG CTGGGGGGGA AAAAAGCCCT GCTGGTCACC    120
GACAAAGGCC TCGGGGCAAT TAAAGATGGC GCGGTGGACA AAACCCTGCA TTATCTGCGG    180
GAGGCCGGGA TCGAGGTGGC GATCTTTGAC GCGTTCGAGC CGAACCCGAA AGACACCAAC    240
GTGCGCGACG GCCTCGCCGT GTTTCGCCGC GAACAGTGCG ACATCATCGT CACCGTGGGC    300
GGCGGCAGCC CGCACGATTG CGGCAAAGGC ATCGGCATCG CCGCCACCCA TGAGGGCGAT    360
CTGTACCACT ATGCCGGAAT CGAGACCCTG ACCAACCCGC TGCCGCCTAT CGTCGCGGTC    420
AATACCACCG CCGGCACCGC CAGCGAGGTC ACCCGCCACT GCGTCCTGAC CAACACCGAA    480

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ACCAAAGTGA AGTTTGTGAT CGTCAGCTGG CGCAAACCTGC CGTCGGTCTC TATCAACGAT 540
CCACTGCTGA TGATCGGTAA ACCGGCCGCC CTGACCGCGG CGACCGGGAT GGATGCCCTG 600
ACCCACGCCG TAGAGGCCTA TATCTCCAAA GACGCTAACC CGGTGACGGA CGCCGCCGCC 660
ATGCAGGCGA TCCGCCTCAT CGCCCGCAAC CTGCGCCAGG CCGTGGCCCT CGGCAGCAAT 720
CTGCAGGCGC GGGAAAACAT GGCCTATGCT TCTCTGCTGG CCGGGATGGC TTTCAATAAC 780
GCCAACCTCG GCTACGTGCA CGCCATGGCG CACCAGCTGG GCGGCCTGTA CGACATGCCG 840
CACGGCGTGG CCAACGCTGT CCTGCTGCCG CATGTGGCGC GCTACAACCT GATCGCCAAC 900
CCGGAGAAAT TCGCCGATAT CGCTGAACTG ATGGGCGAAA ATATCACCGG ACTGTCCACT 960
CTCGACGCGG CGGAAAAAGC CATCGCCGCT ATCACGCGTC TGTCGATGGA TATCGGTATT 1020
CCGCAGCATC TCGCGCATCT GGGGGTAAAA GAGGCCGACT TCCCCTACAT GGCGGAGATG 1080
GCTCTAAAAG ACGGCAATGC GTTCTCGAAC CCGCGTAAAG GCAACGAGCA GGAGATTGCC 1140
GCGATTTTCC GCCAGGCATT CTGA 1164

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(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1380 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: GPD1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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CTTTAATTTT CTTTATCTT ACTCTCCTAC ATAAGACATC AAGAAACAAT TGTATATTGT 60
ACACCCCCCC CCTCCACAAA CACAAATATT GATAATATAA AGATGTCTGC TGCTGCTGAT 120
AGATTAAACT TAACTTCCGG CCACTTGAAT GCTGGTAGAA AGAGAAGTTC CTCTTCTGTT 180
TCTTTGAAGG CTGCCGAAAA GCCTTTCAAG GTTACTGTGA TTGGATCTGG TAACTGGGGT 240
ACTACTATTG CCAAGGTGGT TGCCGAAAAT TGTAAGGGAT ACCCAGAAGT TTTCGCTCCA 300
ATAGTACAAA TGTGGGTGTT CGAAGAAGAG ATCAATGGTG AAAAATTGAC TGAAATCATA 360
AATACTAGAC ATCAAAACGT GAAATACTTG CCTGGCATCA CTCTACCCGA CAATTTGGTT 420
GCTAATCCAG ACTTGATTGA TTCAGTCAAG GATGTCGACA TCATCGTTTT CAACATTCCA 480

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CATCAATTTT TGCCCCGTAT CTGTAGCCAA TTGAAAGGTC ATGTTGATTC ACACGTCAGA 540
 GCTATCTCCT GTCTAAAGGG TTTTGAAGTT GGTGCTAAAG GTGTCCAATT GCTATCCTCT 600
 TACATCACTG AGGAACTAGG TATTCAATGT GGTGCTCTAT CTGGTGCTAA CATTGCCACC 660
 GAAGTCGCTC AAGAACACTG GTCTGAAACA ACAGTTGCTT ACCACATTCC AAAGGATTTT 720
 AGAGGCGAGG GCAAGGACGT CGACCATAAG GTTCTAAAGG CCTTGTTCCA CAGACCTTAC 780
 TTCCACGTTA GTGTCATCGA AGATGTTGCT GGTATCTCCA TCTGTGGTGC TTTGAAGAAC 840
 GTTGTTCCT TAGGTTGTGG TTTCGTCGAA GGTCTAGGCT GGGGTAACAA CGCTTCTGCT 900
 GCCATCCAAA GAGTCGGTTT GGGTGAGATC ATCAGATTCG GTCAAATGTT TTTCCAGAA 960
 TCTAGAGAAG AAACATACTA CCAAGAGTCT GCTGGTGTTG CTGATTTGAT CACCACCTGC 1020
 GCTGGTGGA GAAACGTCAA GGTGCTAGG CTAATGGCTA CTTCTGGTAA GGACGCCTGG 1080
 GAATGTGAAA AGGAGTTGTT GAATGGCCAA TCCGCTCAAG GTTTAATTAC CTGCAAAGAA 1140
 GTTCACGAAT GGTGGAAC ATGTGGCTCT GTCGAAGACT TCCATTATT TGAAGCCGTA 1200
 TACCAAATCG TTTACAACAA CTACCCAATG AAGAACCTGC CGGACATGAT TGAAGAATTA 1260
 GATCTACATG AAGATTAGAT TTATTGGAGA AAGATAACAT ATCATACTTC CCCCCTTTT 1320
 TTCGAGGCTC TTCTATATCA TATTCATAAA TTAGCATTAT GTCATTTCTC ATAACACTT 1380

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2946 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: GPD2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAATTCGAGC CTGAAGTGCT GATTACCTTC AGGTAGACTT CATCTTGACC CATCAACCCC 60
 AGCGTCAATC CTGCAAATAC ACCACCCAGC AGCACTAGGA TGATAGAGAT AATATAGTAC 120
 GTGGTAACGC TTGCCTCATC ACCTACGCTA TGGCCGGAAT CGGCAACATC CCTAGAATTG 180
 AGTACGTGTG ATCCGGATAA CAACGGCAGT GAATATATCT TCGGTATCGT AAAGATGTGA 240
 TATAAGATGA TGTATACCCA ATGAGGAGCG CCTGATCGTG ACCTAGACCT TAGTGGCAAA 300
 AACGACATAT CTATTATAGT GGGGAGAGTT TCGTGCAAAT AACAGACGCA GCAGCAAGTA 360

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ACTGTGACGA TATCAACTCT TTTTTTATTA TGTAATAAGC AAACAAGCAC GAATGGGGAA	420
AGCCTATGTG CAATCACCAA GGTCGTCCCT TTTTCCCAT TTGCTAATTT AGAATTTAAA	480
GAAACCAAAA GAATGAAGAA AGAAAACAAA TACTAGCCCT AACCTGACT TCGTTTCTAT	540
GATAATACCC TGCTTTAATG AACGGTATGC CCTAGGGTAT ATCTCACTCT GTACGTTACA	600
AACTCCGGTT ATTTTATCGG AACATCCGAG CACCCGCGCC TTCCTCAACC CAGGCACCGC	660
CCCAGGTAAC CGTGCGCGAT GAGCTAATCC TGAGCCATCA CCCACCCAC CCGTTGATGA	720
CAGCAATTCG GGAGGGCGAA AATAAACTG GAGCAAGGAA TTACCATCAC CGTCACCATC	780
ACCATCATAT CGCCTTAGCC TCTAGCCATA GCCATCATGC AAGCGTGTAT CTTCTAAGAT	840
TCAGTCATCA TCATTACCGA GTTTGTTTTT CTTACATGA TGAAGAAGGT TTGAGTATGC	900
TCGAAACAAT AAGACGACGA TGGCTCTGCC ATTGGTTATA TTACGCTTTT GCGGCGAGGT	960
GCCGATGGGT TGCTGAGGGG AAGAGTGTTC AGCTTACGGA CCTATTGCCA TTGTTATTCC	1020
GATTAATCTA TTGTTACGCA GCTCTTCTCT ACCCTGTCAT TCTAGTATTT TTTTTTTTTT	1080
TTTTTGTTTT TACTTTTTTT TCTTCTTGCC TTTTTTCTT GTTACTTTTT TTCTAGTTTT	1140
TTTTCCTTCC ACTAAGCTTT TTCCTTGATT TATCCTGGG TTCTTCTTTC TACTCCTTTA	1200
GATTTTTTTT TTATATATTA ATTTTAAAGT TTATGTATTT TGGTAGATTC AATTCTCTTT	1260
CCCTTTCCTT TTCCTTCGCT CCCCTTCCTT ATCAATGCTT GCTGTCAGAA GATTAACAAG	1320
ATACACATTC CTTAAGCGAA CGCATCCGGT GTTATATACT CGTCGTGCAT ATAAAATTTT	1380
GCCTTCAAGA TCTACTTTCC TAAGAAGATC ATTATTACAA ACACAACTGC ACTCAAAGAT	1440
GACTGCTCAT ACTAATATCA AACAGCACAA ACACTGTCAT GAGGACCATC CTATCAGAAG	1500
ATCGGACTCT GCCGTGTCAA TTGTACATTT GAAACGTGCG CCCTTCAAGG TTACAGTGAT	1560
TGGTTCTGGT AACTGGGGGA CCACCATCGC CAAAGTCATT GCGGAAAACA CAGAATTGCA	1620
TTCCCATATC TTCGAGCCAG AGGTGAGAAT GTGGGTTTTT GATGAAAAGA TCGGCGACGA	1680
AAATCTGACG GATATCATAA ATACAAGACA CCAGAACGTT AAATATCTAC CCAATATTGA	1740
CCTGCCCCAT AATCTAGTGG CCGATCCTGA TCTTTTACAC TCCATCAAGG GTGCTGACAT	1800
CCTTGTTTTT AACATCCCTC ATCAATTTTT ACCAAACATA GTCAAACAAT TGCAAGGCCA	1860
CGTGGCCCCT CATGTAAGGG CCATCTCGTG TCTAAAAGGG TTCGAGTTGG GCTCCAAGGG	1920
TGTGCAATTG CTATCCTCCT ATGTTACTGA TGAGTTAGGA ATCCAATGTG GCGCACTATC	1980
TGGTGCAAAC TTGGCACCGG AAGTGGCCAA GGAGCATTGG TCCGAAACCA CCGTGGCTTA	2040

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CCAACTACCA AAGGATTATC AAGGTGATGG CAAGGATGTA GATCATAAGA TTTTGAAATT 2100
 GCTGTTCCAC AGACCTTACT TCCACGTCAA TGTCATCGAT GATGTTGCTG GTATATCCAT 2160
 TGCCGGTGCC TTGAAGAACG TCGTGGCACT TGCATGTGGT TTCGTAGAAG GTATGGGATG 2220
 GGGTAACAAT GCCTCCGCAG CCATTCAAAG GCTGGGTTTA GGTGAAATTA TCAAGTTCGG 2280
 TAGAATGTTT TTCCCAGAAT CCAAAGTCGA GACCTACTAT CAAGAATCCG CTGGTGTTGC 2340
 AGATCTGATC ACCACCTGCT CAGGCGGTAG AAACGTCAAG GTTGCCACAT ACATGGCCAA 2400
 GACCGGTAAG TCAGCCTTGG AAGCAGAAAA GGAATTGCTT AACGGTCAAT CCGCCCAAGG 2460
 GATAATCACA TGCAGAGAAG TTCACGAGTG GCTACAAACA TGTGAGTTGA CCCAAGAATT 2520
 CCCAATTATT CGAGGCAGTC TACCAGATAG TCTACAACAA CGTCCGCATG GAAGACCTAC 2580
 CGGAGATGAT TGAAGAGCTA GACATCGATG ACGAATAGAC ACTCTCCCCC CCCCTCCCCC 2640
 TCTGATCTTT CCTGTTGCCT CTTTTTCCCC CAACCAATTT ATCATTATAC ACAAGTTCTA 2700
 CAACTACTAC TAGTAACATT ACTACAGTTA TTATAATTTT CTATTCTCTT TTTCTTTAAG 2760
 AATCTATCAT TAACGTTAAT TTCTATATAT ACATAACTAC CATTATACAC GCTATTATCG 2820
 TTTACATATC ACATCACCGT TAATGAAAGA TACGACACCC TGTACACTAA CACAATTAAA 2880
 TAATCGCCAT AACCTTTTCT GTTATCTATA GCCCTTAAAG CTGTTTCTTC GAGCTTTTCA 2940
 CTGCAG 2946

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3178 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: GUT2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTGCAGAACT TCGTCTGCTC TGTGCCCCATC CTCGCGGTTA GAAAGAAGCT GAATTGTTTC 60
 ATGCGCAAGG GCATCAGCGA GTGACCAATA ATCACTGCAC TAATTCCTTT TTAGCAACAC 120
 ATACTTATAT ACAGCACCAG ACCTTATGTC TTTTCTCTGC TCCGATACGT TATCCCACCC 180
 AACTTTTATT TCAGTTTGG CAGGGGAAAT TTCACAACCC CGCACGCTAA AAATCGTATT 240

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TAAACTTAAA AGAGAACAGC CACAAATAGG GAACTTTGGT CTAAACGAAG GACTCTCCCT	300
CCCTTATCTT GACCGTGCTA TTGCCATCAC TGCTACAAGA CTAAATACGT ACTAATATAT	360
GTTTTCGGTA ACGAGAAGAA GAGCTGCCGG TGCAGCTGCT GCCATGGCCA CAGCCACGGG	420
GACGCTGTAC TGGATGACTA GCCAAGGTGA TAGGCCGTTA GTGCACAATG ACCCGAGCTA	480
CATGGTGCAA TTCCCCACCG CCGCTCCACC GGCAGGTCTC TAGACGAGAC CTGCTGGACC	540
GTCTGGACAA GACGCATCAA TTCGACGTGT TGATCATCGG TGGCGGGGCC ACGGGGACAG	600
GATGTGCCCT AGATGCTGCG ACCAGGGGAC TCAATGTGGC CCTTGTTGAA AAGGGGGATT	660
TTGCCTCGGG AACGTCGTCC AAATCTACCA AGATGATTCA CGGTGGGGTG CGGTACTTAG	720
AGAAGGCCTT CTGGGAGTTC TCCAAGGCAC AACTGGATCT GGTCATCGAG GCACTCAACG	780
AGCGTAAACA TCTTATCAAC ACTGCCCCTC ACCTGTGCAC GGTGCTACCA ATTCTGATCC	840
CCATCTACAG CACCTGGCAG GTCCCGTACA TCTATATGGG CTGTAAATTC TACGATTTCT	900
TTGGCGGTTT CCAAACTTG AAAAAATCAT ACCTACTGTC CAAATCCGCC ACCGTGGAGA	960
AGGCTCCCAT GCTTACCACA GACAATTTAA AGGCCTCGCT TGTGTACCAT GATGGGTCCT	1020
TTAACGACTC GCGTTTGAAC GCCACTTTAG CCATCACGGG TGTGGAGAAC GGCGCTACCG	1080
TCTTGATCTA TGTCGAGGTA CAAAAATTGA TCAAAGACCC AACTTCTGGT AAGGTTATCG	1140
GTGCCGAGGC CCGGGACGTT GAGACTAATG AGCTTGTCAG AATCAACGCT AAATGTGTGG	1200
TCAATGCCAC GGGCCCATAC AGTGACGCCA TTTTGCAAAT GGACCGCAAC CCATCCGGTC	1260
TGCCGGACTC CCCGCTAAAC GACAACTCCA AGATCAAGTC GACTTTCAAT CAAATCTCCG	1320
TCATGGACCC GAAAATGGTC ATCCCATCTA TTGGCGTTCA CATCGTATTG CCCTCTTTTT	1380
ACTCCCCGAA GGATATGGGT TTGTTGGACG TCAGAACCTC TGATGGCAGA GTGATGTTCT	1440
TTTTACCTTG GCAGGGCAAA GTCCTTGCCG GCACCACAGA CATCCCACTA AAGCAAGTCC	1500
CAGAAAACCC TATGCCTACA GAGGCTGATA TTCAAGATAT CTTGAAAGAA CTACAGCACT	1560
ATATCGAATT CCCCGTGAAA AGAGAAGACG TGCTAAGTGC ATGGGCTGGT GTCAGACCTT	1620
TGGTCAGAGA TCCACGTACA ATCCCCGCAG ACGGGAAGAA GGGCTCTGCC ACTCAGGGCG	1680
TGGTAAGATC CCACTTCTTG TTTACTTCGG ATAATGGCCT AATTACTATT GCAGGTGGTA	1740
AATGGACTAC TTACAGACAA ATGGCTGAGG AAACAGTCGA CAAAGTTGTC GAAGTTGGCG	1800
GATTCCACAA CCTGAAACCT TGTCACACAA GAGATATTAA GCTTGCTGGT GCAGAAGAAT	1860
GGACGCAAAA CTATGTGGCT TTATTGGCTC AAAACTACCA TTTATCATCA AAAATGTCCA	1920

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ACTACTTGGT TCAAACTAC GGAACCCGTT CCTCTATCAT TTGCGAATTT TTCAAAGAAT 1980
 CCATGGAAAA TAAACTGCCT TTGTCCTTAG CCGACAAGGA AAATAACGTA ATCTACTCTA 2040
 GCGAGGAGAA CAACTTGGTC AATTTTGATA CTTTCAGATA TCCATTACACA ATCGGTGAGT 2100
 TAAAGTATTC CATGCAGTAC GAATATTGTA GAACTCCCTT GGACTTCCTT TTAAGAAGAA 2160
 CAAGATTGCG CTTCTTGGAC GCCAAGGAAG CTTTGAATGC CGTGCATGCC ACCGTCAAAG 2220
 TTATGGGTGA TGAGTTCAAT TGGTCGGAGA AAAAGAGGCA GTGGGAACCTT GAAAAAACTG 2280
 TGAACCTCAT CCAAGGACGT TTCGGTGTCT AAATCGATCA TGATAGTTAA GGGTGACAAA 2340
 GATAACATTC ACAAGAGTAA TAATAATGGT AATGATGATA ATAATAATAA TGATAGTAAT 2400
 AACAATAATA ATAATGGTGG TAATGGCAAT GAAATCGCTA TTATTACCTA TTTTCCTTAA 2460
 TGGAAGAGTT AAAGTAACT AAAAAACTA CAAAAATATA TGAAGAAAAA AAAAAAAGA 2520
 GGTAATAGAC TCTACTACTA CAATTGATCT TCAAATTATG ACCTTCCTAG TGTTTATATT 2580
 CTATTTCCAA TACATAATAT AATCTATATA ATCATTGCTG GTAGACTTCC GTTTTAATAT 2640
 CGTTTTAATT ATCCCCTTTA TCTCTAGTCT AGTTTTATCA TAAAATATAG AAACACTAAA 2700
 TAATATTCTT CAAACGGTCC TGGTGCATAC GCAATACATA TTTATGGTGC AAAAAAAAAA 2760
 ATGGAATAAT TTGCTAGTCA TAAACCCTTT CATAAAACAA TACGTAGACA TCGCTACTTG 2820
 AAATTTTCAA GTTTTTATCA GATCCATGTT TCCTATCTGC CTTGACAACC TCATCGTCGA 2880
 AATAGTACCA TTTAGAACGC CCAATATTCA CATTGTGTTT AAGGTCTTTA TTCACCAGTG 2940
 ACGTGTAATG GCCATGATTA ATGTGCCTGT ATGGTTAACC ACTCCAAATA GCTTATATTT 3000
 CATAGTGTCA TTGTTTTTCA ATATAATGTT TAGTATCAAT GGATATGTGA CGACGGTGTT 3060
 ATTTTCTTG GTCAAATCGT AATAAAATCT CGATAAATGG ATGACTAAGA TTTTGGTAA 3120
 AGTTACAAAA TTTATCGTTT TCACTGTTGT CAATTTTTTG TTCTTGTAAT CACTCGAG 3178

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 816 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: GPP1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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ATGAAACGTT TCAATGTTTT AAAATATATC AGAACAACAA AAGCAAATAT ACAAACCATC 60
GCAATGCCTT TGACCACAAA ACCTTTATCT TTGAAAATCA ACGCCGCTCT ATTCGATGTT 120
GACGGTACCA TCATCATCTC TCAACCAGCC ATTGCTGCTT TCTGGAGAGA TTTCGGTAAA 180
GACAAGCCTT ACTTCGATGC CGAACACGTT ATTCACATCT CTCACGGTTG GAGAACTTAC 240
GATGCCATTG CCAAGTTCGC TCCAGACTTT GCTGATGAAG AATACGTAA CAAGCTAGAA 300
GGTGAAATCC CAGAAAAGTA CGGTGAACAC TCCATCGAAG TTCCAGGTGC TGTCAAGTTG 360
TGTAATGCTT TGAACGCCTT GCCAAAGGAA AAATGGGCTG TCGCCACCTC TGGTACCCGT 420
GACATGGCCA AGAAATGGTT CGACATTTTG AAGATCAAGA GACCAGAATA CTTCATCACC 480
GCCAATGATG TCAAGCAAGG TAAGCCTCAC CCAGAACCAT ACTTAAAGGG TAGAAACGGT 540
TTGGGTTTCC CAATTAATGA ACAAGACCCA TCCAAATCTA AGGTGTGTGT CTTTGAAGAC 600
GCACCAGCTG GTATTGCTGC TGGTAAGGCT GCTGGCTGTA AAATCGTTGG TATTGCTACC 660
ACTTTCGATT TGGACTTCTT GAAGGAAAAG GGTGTGACA TCATTGTCAA GAACCACGAA 720
TCTATCAGAG TCGGTGAATA CAACGCTGAA ACCGATGAAG TCGAATTGAT CTTTGATGAC 780
TACTTATACG CTAAGGATGA CTTGTTGAAA TGGTAA 816

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 753 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: GPP2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGGGATTGA CTAATAAACC TCTATCTTTG AAAGTTAACG CCGCTTTGTT CGACGTCGAC 60
GGTACCATTA TCATCTCTCA ACCAGCCATT GCTGCATTCT GGAGGGATTT CGGTAAGGAC 120
AAACCTTATT TCGATGCTGA ACACGTTATC CAAGTCTCGC ATGGTTGGAG AACGTTTGAT 180
GCCATTGCTA AGTTCGCTCC AGACTTTGCC AATGAAGAGT ATGTTAACAA ATTAGAAGCT 240
GAAATTCCGG TCAAGTACGG TGAAAAATCC ATTGAAGTCC CAGGTGCAGT TAAGCTGTGC 300
AACGCTTTGA ACGCTCTACC AAAAGAGAAA TGGGCTGTGG CAACTTCCGG TACCCGTGAT 360

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ATGGCACAAA AATGGTTCGA GCATCTGGGA ATCAGGAGAC CAAAGTACTT CATTACCGCT 420
AATGATGTCA AACAGGGTAA GCCTCATCCA GAACCATATC TGAAGGGCAG GAATGGCTTA 480
GGATATCCGA TCAATGAGCA AGACCCTTCC AAATCTAAGG TAGTAGTATT TGAAGACGCT 540
CCAGCAGGTA TTGCCGCCGG AAAAGCCGCC GGTGTGAAGA TCATTGGTAT TGCCACTACT 600
TTCGACTTGG ACTTCCTAAA GGAAAAAGGC TGTGACATCA TTGTCAAAAA CCACGAATCC 660
ATCAGAGTTG GCGGCTACAA TGCCGAAACA GACGAAGTTG AATTCATTTT TGACGACTAC 720
TTATATGCTA AGGACGATCT GTTGAAATGG TAA 753

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2520 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: GUT1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGTATTGGCC ACGATAACCA CCCTTTGTAT ACTGTTTTTG TTTTTCACAT GGTAAATAAC 60
GACTTTTATT AAACAACGTA TGTA AAAACA TAACAAGAAT CTACCCATAC AGGCCATTTT 120
GTAATTCTTC TCTTCTAATT GGAGTAAAC CATCAATTAA AGGGTGTGGA GTAGCATAGT 180
GAGGGGCTGA CTGCATTGAC AAAAAAATTG AAAAAAAAAA AGGAAAAGGA AAGGAAAAAA 240
AGACAGCCAA GACTTTTAGA ACGGATAAGG TGTAATAAAA TGTGGGGGGA TGCCTGTTCT 300
CGAACCATAT AAAATATACC ATGTGGTTTG AGTTGTGGCC GGAACATAC AAATAGTTAT 360
ATGTTTCCCT CTCTCTCCG ACTTGTAGTA TTCTCCAAAC GTTACATATT CCGATCAAGC 420
CAGCGCCTTT AACTAGTTT AAAACAAGAA CAGAGCCGTA TGTCCAAAAT AATGGAAGAT 480
TTACGAAGTG ACTACGTCCC GCTTATCGCC AGTATTGATG TAGGAACGAC CTCATCCAGA 540
TGCATTCTGT TCAACAGATG GGGCCAGGAC GTTCAAAAC ACCAAATTGA ATATTCAACT 600
TCAGCATCGA AGGGCAAGAT TGGGGTGTCT GGCCTAAGGA GACCCTCTAC AGCCCCAGCT 660
CGTGAAACAC CAAACGCCGG TGACATCAAA ACCAGCGGAA AGCCCATCTT TTCTGCAGAA 720
GGCTATGCCA TTCAAGAAAC CAAATTCCTA AAAATCGAGG AATTGGACTT GGAATTCCAT 780
AACGAACCCA CGTTGAAGTT CCCC AAACCG GGTGGGTTG AGTGCCATCC GCAGAAATTA 840

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CTGGTGAACG	TCGTCCAATG	CCTTGCCTCA	AGTTTGCTCT	CTCTGCAGAC	TATCAACAGC	900
GAACGTGTAG	CAAACGGTCT	CCCACCTTAC	AAGGTAATAT	GCATGGGTAT	AGCAAACATG	960
AGAGAAACCA	CAATTCTGTG	GTCCCGCCGC	ACAGGAAAAC	CAATTGTTAA	CTACGGTATT	1020
GTTTGGAACG	ACACCAGAAC	GATCAAAATC	GTTAGAGACA	AATGGCAAAA	CACTAGCGTC	1080
GATAGGCAAC	TGCAGCTTAG	ACAGAAGACT	GGATTGCCAT	TGCTCTCCAC	GTATTTCTCC	1140
TGTTCCAAGC	TGCGCTGGTT	CCTCGACAAT	GAGCCTCTGT	GTACCAAGGC	GTATGAGGAG	1200
AACGACCTGA	TGTTCGGCAC	TGTGGACACA	TGGCTGATTT	ACCAATTAAC	TAAACAAAAG	1260
GCGTTCGTTT	CTGACGTAAC	CAACGCTTCC	AGAACTGGAT	TTATGAACCT	CTCCACTTTA	1320
AAGTACGACA	ACGAGTTGCT	GGAATTTTGG	GGTATTGACA	AGAACCTGAT	TCACATGCCC	1380
GAAATTGTGT	CCTCATCTCA	ATACTACGGT	GACTTTGGCA	TTCCTGATTG	GATAATGGAA	1440
AAGCTACACG	ATTCGCCAAA	AACAGTACTG	CGAGATCTAG	TCAAGAGAAA	CCTGCCCATA	1500
CAGGGCTGTC	TGGGCGACCA	AAGCGCATCC	ATGGTGGGGC	AACTCGCTTA	CAAACCCGGT	1560
GCTGCAAAAT	GTACTIONTATGG	TACCGGTTGC	TTTTTACTGT	ACAATACGGG	GACCAAAAAA	1620
TTGATCTCCC	AACATGGCGC	ACTGACGACT	CTAGCATTTT	GGTCCCACA	TTTGCAAGAG	1680
TACGGTGGCC	AAAAACCAGA	ATTGAGCAAG	CCACATTTTG	CATTAGAGGG	TTCCGTCGCT	1740
GTGGCTGGTG	CTGTGGTCCA	ATGGCTACGT	GATAATTTAC	GATTGATCGA	TAAATCAGAG	1800
GATGTCGGAC	CGATTGCATC	TACGGTTCCT	GATTCTGGTG	GCGTAGTTTT	CGTCCCCGCA	1860
TTTAGTGGCC	TATTCGCTCC	CTATTGGGAC	CCAGATGCCA	GAGCCACCAT	AATGGGGATG	1920
TCTCAATTCA	CTACTGCCTC	CCACATCGCC	AGAGCTGCCG	TGGAAGGTGT	TTGCTTTCAA	1980
GCCAGGGCTA	TCTTGAAGGC	AATGAGTTCT	GACGCGTTTG	GTGAAGGTTT	CAAAGACAGG	2040
GACTTTTTAG	AGGAAATTTT	CGACGTCACA	TATGAAAAGT	CGCCCCTGTC	GGTTCTGGCA	2100
GTGGATGGCG	GGATGTCGAG	GTCTAATGAA	GTCATGCAAA	TTCAAGCCGA	TATCCTAGGT	2160
CCCTGTGTCA	AAGTCAGAAG	GTCTCCGACA	GCGGAATGTA	CCGCATTGGG	GGCAGCCATT	2220
GCAGCCAATA	TGGCTTTCAA	GGATGTGAAC	GAGCGCCCAT	TATGGAAGGA	CCTACACGAT	2280
GTTAAGAAAT	GGGTCTTTTA	CAATGGAATG	GAGAAAAACG	AACAAATATC	ACCAGAGGCT	2340
CATCCAAACC	TTAAGATATT	CAGAAGTGAA	TCCGACGATG	CTGAAAGGAG	AAAGCATTGG	2400
AAGTATTGGG	AAGTTGCCGT	GGAAAGATCC	AAAGGTGGC	TGAAGGACAT	AGAAGGTGAA	2460
CACGAACAGG	TTCTAGAAAA	CTTCCAATAA	CAACATAAAT	AATTTCTATT	AACAATGTAA	2520

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(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 391 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

(A) ORGANISM: GPD1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

Met Ser Ala Ala Ala Asp Arg Leu Asn Leu Thr Ser Gly His Leu Asn
1           5           10           15
Ala Gly Arg Lys Arg Ser Ser Ser Ser Val Ser Leu Lys Ala Ala Glu
20           25           30
Lys Pro Phe Lys Val Thr Val Ile Gly Ser Gly Asn Trp Gly Thr Thr
35           40           45
Ile Ala Lys Val Val Ala Glu Asn Cys Lys Gly Tyr Pro Glu Val Phe
50           55           60
Ala Pro Ile Val Gln Met Trp Val Phe Glu Glu Glu Ile Asn Gly Glu
65           70           75           80
Lys Leu Thr Glu Ile Ile Asn Thr Arg His Gln Asn Val Lys Tyr Leu
85           90           95
Pro Gly Ile Thr Leu Pro Asp Asn Leu Val Ala Asn Pro Asp Leu Ile
100          105          110
Asp Ser Val Lys Asp Val Asp Ile Ile Val Phe Asn Ile Pro His Gln
115          120          125
Phe Leu Pro Arg Ile Cys Ser Gln Leu Lys Gly His Val Asp Ser His
130          135          140
Val Arg Ala Ile Ser Cys Leu Lys Gly Phe Glu Val Gly Ala Lys Gly
145          150          155          160
Val Gln Leu Leu Ser Ser Tyr Ile Thr Glu Glu Leu Gly Ile Gln Cys
165          170          175
Gly Ala Leu Ser Gly Ala Asn Ile Ala Thr Glu Val Ala Gln Glu His
180          185          190
Trp Ser Glu Thr Thr Val Ala Tyr His Ile Pro Lys Asp Phe Arg Gly
195          200          205
Glu Gly Lys Asp Val Asp His Lys Val Leu Lys Ala Leu Phe His Arg

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210	215	220
Pro Tyr Phe His Val Ser Val Ile Glu Asp Val Ala Gly Ile Ser Ile		
225	230	235 240
Cys Gly Ala Leu Lys Asn Val Val Ala Leu Gly Cys Gly Phe Val Glu		
	245	250 255
Gly Leu Gly Trp Gly Asn Asn Ala Ser Ala Ala Ile Gln Arg Val Gly		
	260	265 270
Leu Gly Glu Ile Ile Arg Phe Gly Gln Met Phe Phe Pro Glu Ser Arg		
	275	280 285
Glu Glu Thr Tyr Tyr Gln Glu Ser Ala Gly Val Ala Asp Leu Ile Thr		
	290	295 300
Thr Cys Ala Gly Gly Arg Asn Val Lys Val Ala Arg Leu Met Ala Thr		
	305	310 315 320
Ser Gly Lys Asp Ala Trp Glu Cys Glu Lys Glu Leu Leu Asn Gly Gln		
	325	330 335
Ser Ala Gln Gly Leu Ile Thr Cys Lys Glu Val His Glu Trp Leu Glu		
	340	345 350
Thr Cys Gly Ser Val Glu Asp Phe Pro Leu Phe Glu Ala Val Tyr Gln		
	355	360 365
Ile Val Tyr Asn Asn Tyr Pro Met Lys Asn Leu Pro Asp Met Ile Glu		
	370	375 380
Glu Leu Asp Leu His Glu Asp		
385	390	

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 384 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: GPD2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Thr Ala His Thr Asn Ile Lys Gln His Lys His Cys His Glu Asp
1 5 10 15
His Pro Ile Arg Arg Ser Asp Ser Ala Val Ser Ile Val His Leu Lys
20 25 30

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Arg Ala Pro Phe Lys Val Thr Val Ile Gly Ser Gly Asn Trp Gly Thr
 35 40 45
 Thr Ile Ala Lys Val Ile Ala Glu Asn Thr Glu Leu His Ser His Ile
 50 55 60
 Phe Glu Pro Glu Val Arg Met Trp Val Phe Asp Glu Lys Ile Gly Asp
 65 70 75 80
 Glu Asn Leu Thr Asp Ile Ile Asn Thr Arg His Gln Asn Val Lys Tyr
 85 90 95
 Leu Pro Asn Ile Asp Leu Pro His Asn Leu Val Ala Asp Pro Asp Leu
 100 105 110
 Leu His Ser Ile Lys Gly Ala Asp Ile Leu Val Phe Asn Ile Pro His
 115 120 125
 Gln Phe Leu Pro Asn Ile Val Lys Gln Leu Gln Gly His Val Ala Pro
 130 135 140
 His Val Arg Ala Ile Ser Cys Leu Lys Gly Phe Glu Leu Gly Ser Lys
 145 150 155 160
 Gly Val Gln Leu Leu Ser Ser Tyr Val Thr Asp Glu Leu Gly Ile Gln
 165 170 175
 Cys Gly Ala Leu Ser Gly Ala Asn Leu Ala Pro Glu Val Ala Lys Glu
 180 185 190
 His Trp Ser Glu Thr Thr Val Ala Tyr Gln Leu Pro Lys Asp Tyr Gln
 195 200 205
 Gly Asp Gly Lys Asp Val Asp His Lys Ile Leu Lys Leu Leu Phe His
 210 215 220
 Arg Pro Tyr Phe His Val Asn Val Ile Asp Asp Val Ala Gly Ile Ser
 225 230 235 240
 Ile Ala Gly Ala Leu Lys Asn Val Val Ala Leu Ala Cys Gly Phe Val
 245 250 255
 Glu Gly Met Gly Trp Gly Asn Asn Ala Ser Ala Ala Ile Gln Arg Leu
 260 265 270
 Gly Leu Gly Glu Ile Ile Lys Phe Gly Arg Met Phe Phe Pro Glu Ser
 275 280 285
 Lys Val Glu Thr Tyr Tyr Gln Glu Ser Ala Gly Val Ala Asp Leu Ile
 290 295 300
 Thr Thr Cys Ser Gly Gly Arg Asn Val Lys Val Ala Thr Tyr Met Ala
 305 310 315 320
 Lys Thr Gly Lys Ser Ala Leu Glu Ala Glu Lys Glu Leu Leu Asn Gly
 325 330 335

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Gln Ser Ala Gln Gly Ile Ile Thr Cys Arg Glu Val His Glu Trp Leu
 340 345 350

Gln Thr Cys Glu Leu Thr Gln Glu Phe Pro Ile Ile Arg Gly Ser Leu
 355 360 365

Pro Asp Ser Leu Gln Gln Arg Pro His Gly Arg Pro Thr Gly Asp Asp
 370 375 380

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 614 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: GUT2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Met Thr Arg Ala Thr Trp Cys Asn Ser Pro Pro Pro Leu His Arg Gln
 1 5 10 15

Val Ser Arg Arg Asp Leu Leu Asp Arg Leu Asp Lys Thr His Gln Phe
 20 25 30

Asp Val Leu Ile Ile Gly Gly Gly Ala Thr Gly Thr Gly Cys Ala Leu
 35 40 45

Asp Ala Ala Thr Arg Gly Leu Asn Val Ala Leu Val Glu Lys Gly Asp
 50 55 60

Phe Ala Ser Gly Thr Ser Ser Lys Ser Thr Lys Met Ile His Gly Gly
 65 70 75 80

Val Arg Tyr Leu Glu Lys Ala Phe Trp Glu Phe Ser Lys Ala Gln Leu
 85 90 95

Asp Leu Val Ile Glu Ala Leu Asn Glu Arg Lys His Leu Ile Asn Thr
 100 105 110

Ala Pro His Leu Cys Thr Val Leu Pro Ile Leu Ile Pro Ile Tyr Ser
 115 120 125

Thr Trp Gln Val Pro Tyr Ile Tyr Met Gly Cys Lys Phe Tyr Asp Phe
 130 135 140

Phe Gly Gly Ser Gln Asn Leu Lys Lys Ser Tyr Leu Leu Ser Lys Ser
 145 150 155 160

Ala Thr Val Glu Lys Ala Pro Met Leu Thr Thr Asp Asn Leu Lys Ala

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					165					170							175
Ser	Leu	Val	Tyr	His	Asp	Gly	Ser	Phe	Asn	Asp	Ser	Arg	Leu	Asn	Ala		
			180					185					190				
Thr	Leu	Ala	Ile	Thr	Gly	Val	Glu	Asn	Gly	Ala	Thr	Val	Leu	Ile	Tyr		
		195					200					205					
Val	Glu	Val	Gln	Lys	Leu	Ile	Lys	Asp	Pro	Thr	Ser	Gly	Lys	Val	Ile		
	210					215					220						
Gly	Ala	Glu	Ala	Arg	Asp	Val	Glu	Thr	Asn	Glu	Leu	Val	Arg	Ile	Asn		
225					230					235					240		
Ala	Lys	Cys	Val	Val	Asn	Ala	Thr	Gly	Pro	Tyr	Ser	Asp	Ala	Ile	Leu		
				245					250					255			
Gln	Met	Asp	Arg	Asn	Pro	Ser	Gly	Leu	Pro	Asp	Ser	Pro	Leu	Asn	Asp		
			260					265					270				
Asn	Ser	Lys	Ile	Lys	Ser	Thr	Phe	Asn	Gln	Ile	Ser	Val	Met	Asp	Pro		
		275					280					285					
Lys	Met	Val	Ile	Pro	Ser	Ile	Gly	Val	His	Ile	Val	Leu	Pro	Ser	Phe		
	290					295					300						
Tyr	Ser	Pro	Lys	Asp	Met	Gly	Leu	Leu	Asp	Val	Arg	Thr	Ser	Asp	Gly		
305					310					315					320		
Arg	Val	Met	Phe	Phe	Leu	Pro	Trp	Gln	Gly	Lys	Val	Leu	Ala	Gly	Thr		
				325					330					335			
Thr	Asp	Ile	Pro	Leu	Lys	Gln	Val	Pro	Glu	Asn	Pro	Met	Pro	Thr	Glu		
			340					345					350				
Ala	Asp	Ile	Gln	Asp	Ile	Leu	Lys	Glu	Leu	Gln	His	Tyr	Ile	Glu	Phe		
		355					360					365					
Pro	Val	Lys	Arg	Glu	Asp	Val	Leu	Ser	Ala	Trp	Ala	Gly	Val	Arg	Pro		
	370					375					380						
Leu	Val	Arg	Asp	Pro	Arg	Thr	Ile	Pro	Ala	Asp	Gly	Lys	Lys	Gly	Ser		
385					390					395					400		
Ala	Thr	Gln	Gly	Val	Val	Arg	Ser	His	Phe	Leu	Phe	Thr	Ser	Asp	Asn		
				405					410					415			
Gly	Leu	Ile	Thr	Ile	Ala	Gly	Gly	Lys	Trp	Thr	Thr	Tyr	Arg	Gln	Met		
			420					425					430				
Ala	Glu	Glu	Thr	Val	Asp	Lys	Val	Val	Glu	Val	Gly	Gly	Phe	His	Asn		
			435				440					445					
Leu	Lys	Pro	Cys	His	Thr	Arg	Asp	Ile	Lys	Leu	Ala	Gly	Ala	Glu	Glu		
	450					455					460						

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Trp Thr Gln Asn Tyr Val Ala Leu Leu Ala Gln Asn Tyr His Leu Ser
 465 470 475 480
 Ser Lys Met Ser Asn Tyr Leu Val Gln Asn Tyr Gly Thr Arg Ser Ser
 485 490 495
 Ile Ile Cys Glu Phe Phe Lys Glu Ser Met Glu Asn Lys Leu Pro Leu
 500 505 510
 Ser Leu Ala Asp Lys Glu Asn Asn Val Ile Tyr Ser Ser Glu Glu Asn
 515 520 525
 Asn Leu Val Asn Phe Asp Thr Phe Arg Tyr Pro Phe Thr Ile Gly Glu
 530 535 540
 Leu Lys Tyr Ser Met Gln Tyr Glu Tyr Cys Arg Thr Pro Leu Asp Phe
 545 550 555 560
 Leu Leu Arg Arg Thr Arg Phe Ala Phe Leu Asp Ala Lys Glu Ala Leu
 565 570 575
 Asn Ala Val His Ala Thr Val Lys Val Met Gly Asp Glu Phe Asn Trp
 580 585 590
 Ser Glu Lys Lys Arg Gln Trp Glu Leu Glu Lys Thr Val Asn Phe Ile
 595 600 605
 Gln Gly Arg Phe Gly Val
 610

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 339 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

(A) ORGANISM: GPSA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Asn Gln Arg Asn Ala Ser Met Thr Val Ile Gly Ala Gly Ser Tyr
 1 5 10 15
 Gly Thr Ala Leu Ala Ile Thr Leu Ala Arg Asn Gly His Glu Val Val
 20 25 30
 Leu Trp Gly His Asp Pro Glu His Ile Ala Thr Leu Glu Arg Asp Arg
 35 40 45
 Cys Asn Ala Ala Phe Leu Pro Asp Val Pro Phe Pro Asp Thr Leu His
 50 55 60

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Leu Glu Ser Asp Leu Ala Thr Ala Leu Ala Ala Ser Arg Asn Ile Leu
 65 70 75 80
 Val Val Val Pro Ser His Val Phe Gly Glu Val Leu Arg Gln Ile Lys
 85 90 95
 Pro Leu Met Arg Pro Asp Ala Arg Leu Val Trp Ala Thr Lys Gly Leu
 100 105 110
 Glu Ala Glu Thr Gly Arg Leu Leu Gln Asp Val Ala Arg Glu Ala Leu
 115 120 125
 Gly Asp Gln Ile Pro Leu Ala Val Ile Ser Gly Pro Thr Phe Ala Lys
 130 135 140
 Glu Leu Ala Ala Gly Leu Pro Thr Ala Ile Ser Leu Ala Ser Thr Asp
 145 150 155 160
 Gln Thr Phe Ala Asp Asp Leu Gln Gln Leu Leu His Cys Gly Lys Ser
 165 170 175
 Phe Arg Val Tyr Ser Asn Pro Asp Phe Ile Gly Val Gln Leu Gly Gly
 180 185 190
 Ala Val Lys Asn Val Ile Ala Ile Gly Ala Gly Met Ser Asp Gly Ile
 195 200 205
 Gly Phe Gly Ala Asn Ala Arg Thr Ala Leu Ile Thr Arg Gly Leu Ala
 210 215 220
 Glu Met Ser Arg Leu Gly Ala Ala Leu Gly Ala Asp Pro Ala Thr Phe
 225 230 235 240
 Met Gly Met Ala Gly Leu Gly Asp Leu Val Leu Thr Cys Thr Asp Asn
 245 250 255
 Gln Ser Arg Asn Arg Arg Phe Gly Met Met Leu Gly Gln Gly Met Asp
 260 265 270
 Val Gln Ser Ala Gln Glu Lys Ile Gly Gln Val Val Glu Gly Tyr Arg
 275 280 285
 Asn Thr Lys Glu Val Arg Glu Leu Ala His Arg Phe Gly Val Glu Met
 290 295 300
 Pro Ile Thr Glu Glu Ile Tyr Gln Val Leu Tyr Cys Gly Lys Asn Ala
 305 310 315 320
 Arg Glu Ala Ala Leu Thr Leu Leu Gly Arg Ala Arg Lys Asp Glu Arg
 325 330 335
 Ser Ser His

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- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 501 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: unknown
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: GLPD

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```

Met Glu Thr Lys Asp Leu Ile Val Ile Gly Gly Gly Ile Asn Gly Ala
1           5           10           15
Gly Ile Ala Ala Asp Ala Ala Gly Arg Gly Leu Ser Val Leu Met Leu
20           25           30
Glu Ala Gln Asp Leu Ala Cys Ala Thr Ser Ser Ala Ser Ser Lys Leu
35           40           45
Ile His Gly Gly Leu Arg Tyr Leu Glu His Tyr Glu Phe Arg Leu Val
50           55           60
Ser Glu Ala Leu Ala Glu Arg Glu Val Leu Leu Lys Met Ala Pro His
65           70           75           80
Ile Ala Phe Pro Met Arg Phe Arg Leu Pro His Arg Pro His Leu Arg
85           90           95
Pro Ala Trp Met Ile Arg Ile Gly Leu Phe Met Tyr Asp His Leu Gly
100          105          110
Lys Arg Thr Ser Leu Pro Gly Ser Thr Gly Leu Arg Phe Gly Ala Asn
115          120          125
Ser Val Leu Lys Pro Glu Ile Lys Arg Gly Phe Glu Tyr Ser Asp Cys
130          135          140
Trp Val Asp Asp Ala Arg Leu Val Leu Ala Asn Ala Gln Met Val Val
145          150          155          160
Arg Lys Gly Gly Glu Val Leu Thr Arg Thr Arg Ala Thr Ser Ala Arg
165          170          175
Arg Glu Asn Gly Leu Trp Ile Val Glu Ala Glu Asp Ile Asp Thr Gly
180          185          190
Lys Lys Tyr Ser Trp Gln Ala Arg Gly Leu Val Asn Ala Thr Gly Pro
195          200          205
Trp Val Lys Gln Phe Phe Asp Asp Gly Met His Leu Pro Ser Pro Tyr
210          215          220

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Gly Ile Arg Leu Ile Lys Gly Ser His Ile Val Val Pro Arg Val His
 225 230 235 240
 Thr Gln Lys Gln Ala Tyr Ile Leu Gln Asn Glu Asp Lys Arg Ile Val
 245 250 255
 Phe Val Ile Pro Trp Met Asp Glu Phe Ser Ile Ile Gly Thr Thr Asp
 260 265 270
 Val Glu Tyr Lys Gly Asp Pro Lys Ala Val Lys Ile Glu Glu Ser Glu
 275 280 285
 Ile Asn Tyr Leu Leu Asn Val Tyr Asn Thr His Phe Lys Lys Gln Leu
 290 295 300
 Ser Arg Asp Asp Ile Val Trp Thr Tyr Ser Gly Val Arg Pro Leu Cys
 305 310 315 320
 Asp Asp Glu Ser Asp Ser Pro Gln Ala Ile Thr Arg Asp Tyr Thr Leu
 325 330 335
 Asp Ile His Asp Glu Asn Gly Lys Ala Pro Leu Leu Ser Val Phe Gly
 340 345 350
 Gly Lys Leu Thr Thr Tyr Arg Lys Leu Ala Glu His Ala Leu Glu Lys
 355 360 365
 Leu Thr Pro Tyr Tyr Gln Gly Ile Gly Pro Ala Trp Thr Lys Glu Ser
 370 375 380
 Val Leu Pro Gly Gly Ala Ile Glu Gly Asp Arg Asp Asp Tyr Ala Ala
 385 390 395 400
 Arg Leu Arg Arg Arg Tyr Pro Phe Leu Thr Glu Ser Leu Ala Arg His
 405 410 415
 Tyr Ala Arg Thr Tyr Gly Ser Asn Ser Glu Leu Leu Leu Gly Asn Ala
 420 425 430
 Gly Thr Val Ser Asp Leu Gly Glu Asp Phe Gly His Glu Phe Tyr Glu
 435 440 445
 Ala Glu Leu Lys Tyr Leu Val Asp His Glu Trp Val Arg Arg Ala Asp
 450 455 460
 Asp Ala Leu Trp Arg Arg Thr Lys Gln Gly Met Trp Leu Asn Ala Asp
 465 470 475 480
 Gln Gln Ser Arg Val Ser Gln Trp Leu Val Glu Tyr Thr Gln Gln Arg
 485 490 495
 Leu Ser Leu Ala Ser
 500

(2) INFORMATION FOR SEQ ID NO:16:

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(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 542 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: GLPABC

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```

Met Lys Thr Arg Asp Ser Gln Ser Ser Asp Val Ile Ile Ile Gly Gly
1           5           10           15

Gly Ala Thr Gly Ala Gly Ile Ala Arg Asp Cys Ala Leu Arg Gly Leu
20           25           30

Arg Val Ile Leu Val Glu Arg His Asp Ile Ala Thr Gly Ala Thr Gly
35           40           45

Arg Asn His Gly Leu Leu His Ser Gly Ala Arg Tyr Ala Val Thr Asp
50           55           60

Ala Glu Ser Ala Arg Glu Cys Ile Ser Glu Asn Gln Ile Leu Lys Arg
65           70           75           80

Ile Ala Arg His Cys Val Glu Pro Thr Asn Gly Leu Phe Ile Thr Leu
85           90           95

Pro Glu Asp Asp Leu Ser Phe Gln Ala Thr Phe Ile Arg Ala Cys Glu
100          105          110

Glu Ala Gly Ile Ser Ala Glu Ala Ile Asp Pro Gln Gln Ala Arg Ile
115          120          125

Ile Glu Pro Ala Val Asn Pro Ala Leu Ile Gly Ala Val Lys Val Pro
130          135          140

Asp Gly Thr Val Asp Pro Phe Arg Leu Thr Ala Ala Asn Met Leu Asp
145          150          155          160

Ala Lys Glu His Gly Ala Val Ile Leu Thr Ala His Glu Val Thr Gly
165          170          175

Leu Ile Arg Glu Gly Ala Thr Val Cys Gly Val Arg Val Arg Asn His
180          185          190

Leu Thr Gly Glu Thr Gln Ala Leu His Ala Pro Val Val Val Asn Ala
195          200          205

Ala Gly Ile Trp Gly Gln His Ile Ala Glu Tyr Ala Asp Leu Arg Ile
210          215          220

Arg Met Phe Pro Ala Lys Gly Ser Leu Leu Ile Met Asp His Arg Ile

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225		230		235		240
Asn Gln His Val	Ile Asn Arg Cys Arg Lys Pro Ser Asp Ala Asp Ile					
	245			250		255
Leu Val Pro Gly Asp Thr Ile Ser Leu Ile Gly Thr Thr Ser Leu Arg						
	260			265		270
Ile Asp Tyr Asn Glu Ile Asp Asp Asn Arg Val Thr Ala Glu Glu Val						
	275			280		285
Asp Ile Leu Leu Arg Glu Gly Glu Lys Leu Ala Pro Val Met Ala Lys						
	290			295		300
Thr Arg Ile Leu Arg Ala Tyr Ser Gly Val Arg Pro Leu Val Ala Ser						
	305			310		315
Asp Asp Asp Pro Ser Gly Arg Asn Leu Ser Arg Gly Ile Val Leu Leu						
	325			330		335
Asp His Ala Glu Arg Asp Gly Leu Asp Gly Phe Ile Thr Ile Thr Gly						
	340			345		350
Gly Lys Leu Met Thr Tyr Arg Leu Met Ala Glu Trp Ala Thr Asp Ala						
	355			360		365
Val Cys Arg Lys Leu Gly Asn Thr Arg Pro Cys Thr Thr Ala Asp Leu						
	370			375		380
Ala Leu Pro Gly Ser Gln Glu Pro Ala Glu Val Thr Leu Arg Lys Val						
	385			390		395
Ile Ser Leu Pro Ala Pro Leu Arg Gly Ser Ala Val Tyr Arg His Gly						
	405			410		415
Asp Arg Thr Pro Ala Trp Leu Ser Glu Gly Arg Leu His Arg Ser Leu						
	420			425		430
Val Cys Glu Cys Glu Ala Val Thr Ala Gly Glu Val Gln Tyr Ala Val						
	435			440		445
Glu Asn Leu Asn Val Asn Ser Leu Leu Asp Leu Arg Arg Arg Thr Arg						
	450			455		460
Val Gly Met Gly Thr Cys Gln Gly Glu Leu Cys Ala Cys Arg Ala Ala						
	465			470		475
Gly Leu Leu Gln Arg Phe Asn Val Thr Thr Ser Ala Gln Ser Ile Glu						
	485			490		495
Gln Leu Ser Thr Phe Leu Asn Glu Arg Trp Lys Gly Val Gln Pro Ile						
	500			505		510
Ala Trp Gly Asp Ala Leu Arg Glu Ser Glu Phe Thr Arg Trp Val Tyr						
	515			520		525

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Gln Gly Leu Cys Gly Leu Glu Lys Glu Gln Lys Asp Ala Leu
 530 535 540

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 250 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: unknown
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: GPP2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Gly Leu Thr Thr Lys Pro Leu Ser Leu Lys Val Asn Ala Ala Leu
 1 5 10 15

Phe Asp Val Asp Gly Thr Ile Ile Ile Ser Gln Pro Ala Ile Ala Ala
 20 25 30

Phe Trp Arg Asp Phe Gly Lys Asp Lys Pro Tyr Phe Asp Ala Glu His
 35 40 45

Val Ile Gln Val Ser His Gly Trp Arg Thr Phe Asp Ala Ile Ala Lys
 50 55 60

Phe Ala Pro Asp Phe Ala Asn Glu Glu Tyr Val Asn Lys Leu Glu Ala
 65 70 75 80

Glu Ile Pro Val Lys Tyr Gly Glu Lys Ser Ile Glu Val Pro Gly Ala
 85 90 95

Val Lys Leu Cys Asn Ala Leu Asn Ala Leu Pro Lys Glu Lys Trp Ala
 100 105 110

Val Ala Thr Ser Gly Thr Arg Asp Met Ala Gln Lys Trp Phe Glu His
 115 120 125

Leu Gly Ile Arg Arg Pro Lys Tyr Phe Ile Thr Ala Asn Asp Val Lys
 130 135 140

Gln Gly Lys Pro His Pro Glu Pro Tyr Leu Lys Gly Arg Asn Gly Leu
 145 150 155 160

Gly Tyr Pro Ile Asn Glu Gln Asp Pro Ser Lys Ser Lys Val Val Val
 165 170 175

Phe Glu Asp Ala Pro Ala Gly Ile Ala Ala Gly Lys Ala Ala Gly Cys
 180 185 190

Lys Ile Ile Gly Ile Ala Thr Thr Phe Asp Leu Asp Phe Leu Lys Glu
 195 200 205

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Lys Gly Cys Asp Ile Ile Val Lys Asn His Glu Ser Ile Arg Val Gly
 210 215 220

Gly Tyr Asn Ala Glu Thr Asp Glu Val Glu Phe Ile Phe Asp Asp Tyr
 225 230 235 240

Leu Tyr Ala Lys Asp Asp Leu Leu Lys Trp
 245 250

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 709 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: unknown
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: GUT1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met Phe Pro Ser Leu Phe Arg Leu Val Val Phe Ser Lys Arg Tyr Ile
 1 5 10 15

Phe Arg Ser Ser Gln Arg Leu Tyr Thr Ser Leu Lys Gln Glu Gln Ser
 20 25 30

Arg Met Ser Lys Ile Met Glu Asp Leu Arg Ser Asp Tyr Val Pro Leu
 35 40 45

Ile Ala Ser Ile Asp Val Gly Thr Thr Ser Ser Arg Cys Ile Leu Phe
 50 55 60

Asn Arg Trp Gly Gln Asp Val Ser Lys His Gln Ile Glu Tyr Ser Thr
 65 70 75 80

Ser Ala Ser Lys Gly Lys Ile Gly Val Ser Gly Leu Arg Arg Pro Ser
 85 90 95

Thr Ala Pro Ala Arg Glu Thr Pro Asn Ala Gly Asp Ile Lys Thr Ser
 100 105 110

Gly Lys Pro Ile Phe Ser Ala Glu Gly Tyr Ala Ile Gln Glu Thr Lys
 115 120 125

Phe Leu Lys Ile Glu Glu Leu Asp Leu Asp Phe His Asn Glu Pro Thr
 130 135 140

Leu Lys Phe Pro Lys Pro Gly Trp Val Glu Cys His Pro Gln Lys Leu
 145 150 155 160

Leu Val Asn Val Val Gln Cys Leu Ala Ser Ser Leu Leu Ser Leu Gln

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				165					170					175			
Thr	Ile	Asn	Ser	Glu	Arg	Val	Ala	Asn	Gly	Leu	Pro	Pro	Tyr	Lys	Val		
			180					185					190				
Ile	Cys	Met	Gly	Ile	Ala	Asn	Met	Arg	Glu	Thr	Thr	Ile	Leu	Trp	Ser		
		195					200					205					
Arg	Arg	Thr	Gly	Lys	Pro	Ile	Val	Asn	Tyr	Gly	Ile	Val	Trp	Asn	Asp		
	210					215					220						
Thr	Arg	Thr	Ile	Lys	Ile	Val	Arg	Asp	Lys	Trp	Gln	Asn	Thr	Ser	Val		
225					230					235					240		
Asp	Arg	Gln	Leu	Gln	Leu	Arg	Gln	Lys	Thr	Gly	Leu	Pro	Leu	Leu	Ser		
			245					250						255			
Thr	Tyr	Phe	Ser	Cys	Ser	Lys	Leu	Arg	Trp	Phe	Leu	Asp	Asn	Glu	Pro		
			260					265					270				
Leu	Cys	Thr	Lys	Ala	Tyr	Glu	Glu	Asn	Asp	Leu	Met	Phe	Gly	Thr	Val		
	275						280					285					
Asp	Thr	Trp	Leu	Ile	Tyr	Gln	Leu	Thr	Lys	Gln	Lys	Ala	Phe	Val	Ser		
	290					295					300						
Asp	Val	Thr	Asn	Ala	Ser	Arg	Thr	Gly	Phe	Met	Asn	Leu	Ser	Thr	Leu		
305				310					315						320		
Lys	Tyr	Asp	Asn	Glu	Leu	Leu	Glu	Phe	Trp	Gly	Ile	Asp	Lys	Asn	Leu		
			325					330						335			
Ile	His	Met	Pro	Glu	Ile	Val	Ser	Ser	Ser	Gln	Tyr	Tyr	Gly	Asp	Phe		
		340					345						350				
Gly	Ile	Pro	Asp	Trp	Ile	Met	Glu	Lys	Leu	His	Asp	Ser	Pro	Lys	Thr		
	355						360					365					
Val	Leu	Arg	Asp	Leu	Val	Lys	Arg	Asn	Leu	Pro	Ile	Gln	Gly	Cys	Leu		
	370					375					380						
Gly	Asp	Gln	Ser	Ala	Ser	Met	Val	Gly	Gln	Leu	Ala	Tyr	Lys	Pro	Gly		
385				390					395						400		
Ala	Ala	Lys	Cys	Thr	Tyr	Gly	Thr	Gly	Cys	Phe	Leu	Leu	Tyr	Asn	Thr		
			405					410						415			
Gly	Thr	Lys	Lys	Leu	Ile	Ser	Gln	His	Gly	Ala	Leu	Thr	Thr	Leu	Ala		
		420					425						430				
Phe	Trp	Phe	Pro	His	Leu	Gln	Glu	Tyr	Gly	Gly	Gln	Lys	Pro	Glu	Leu		
	435						440					445					
Ser	Lys	Pro	His	Phe	Ala	Leu	Glu	Gly	Ser	Val	Ala	Val	Ala	Gly	Ala		
450						455					460						

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Val Val Gln Trp Leu Arg Asp Asn Leu Arg Leu Ile Asp Lys Ser Glu
 465 470 475 480
 Asp Val Gly Pro Ile Ala Ser Thr Val Pro Asp Ser Gly Gly Val Val
 485 490 495
 Phe Val Pro Ala Phe Ser Gly Leu Phe Ala Pro Tyr Trp Asp Pro Asp
 500 505 510
 Ala Arg Ala Thr Ile Met Gly Met Ser Gln Phe Thr Thr Ala Ser His
 515 520 525
 Ile Ala Arg Ala Ala Val Glu Gly Val Cys Phe Gln Ala Arg Ala Ile
 530 535 540
 Leu Lys Ala Met Ser Ser Asp Ala Phe Gly Glu Gly Ser Lys Asp Arg
 545 550 555 560
 Asp Phe Leu Glu Glu Ile Ser Asp Val Thr Tyr Glu Lys Ser Pro Leu
 565 570 575
 Ser Val Leu Ala Val Asp Gly Gly Met Ser Arg Ser Asn Glu Val Met
 580 585 590
 Gln Ile Gln Ala Asp Ile Leu Gly Pro Cys Val Lys Val Arg Arg Ser
 595 600 605
 Pro Thr Ala Glu Cys Thr Ala Leu Gly Ala Ala Ile Ala Ala Asn Met
 610 615 620
 Ala Phe Lys Asp Val Asn Glu Arg Pro Leu Trp Lys Asp Leu His Asp
 625 630 635 640
 Val Lys Lys Trp Val Phe Tyr Asn Gly Met Glu Lys Asn Glu Gln Ile
 645 650 655
 Ser Pro Glu Ala His Pro Asn Leu Lys Ile Phe Arg Ser Glu Ser Asp
 660 665 670
 Asp Ala Glu Arg Arg Lys His Trp Lys Tyr Trp Glu Val Ala Val Glu
 675 680 685
 Arg Ser Lys Gly Trp Leu Lys Asp Ile Glu Gly Glu His Glu Gln Val
 690 695 700
 Leu Glu Asn Phe Gln
 705

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 12145 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: PHK28-26

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTCGACCACC	ACGGTGGTGA	CTTTAATGCC	GCTCTCATGC	AGCAGCTCGG	TGGCGGTCTC	60
AAAATTCAGG	ATGTCGCCCG	TATAGTTTTT	GATAATCAGC	AAGACGCCTT	CGCCGCCGTC	120
AATTTGCATC	GCGCATTCAA	ACATTTTGTC	CGGCGTCGGC	GAGGTGAATA	TTTCCCCCGG	180
ACAGGCGCCG	GAGAGCATGC	CCTGGCCGAT	ATAGCCGCAG	TGCATCGGTT	CATGTCCGCT	240
GCCGCCGCCG	GAGAGCAGGG	CCACCTTGCC	AGCCACCGGC	GCGTCGGTGC	GGGTCACATA	300
CAGCGGGTCC	TGATGCAGGG	TCAGCTGCGG	ATGGGCTTTA	GCCAGCCCCT	GTAATTGTTC	360
ATTCAGTACA	TCTTCAACAC	GGTTAATCAG	CTTTTTTATT	ATTCAGTGCT	CCGTTGGAGA	420
AGGTTTCGATG	CCGCCTCTCT	GCTGGCGGAG	GCGGTCATCG	CGTAGGGGTA	TCGTCTGACG	480
GTGGAGCGTG	CCTGGCGATA	TGATGATTCT	GGCTGAGCGG	ACGAAAAAAAA	GAATGCCCCG	540
ACGATCGGGT	TTCATTACGA	AACATTGCTT	CCTGATTTTG	TTTCTTTATG	GAACGTTTTT	600
GCTGAGGATA	TGGTGAAAAT	GCGAGCTGGC	GCGCTTTTTT	TCTTCTGCCA	TAAGCGGCGG	660
TCAGGATAGC	CGGCGAAGCG	GGTGGGAAAA	AATTTTTTGC	TGATTTTCTG	CCGACTGCGG	720
GAGAAAAGGC	GGTCAAACAC	GGAGGATTGT	AAGGGCATTG	TGCGGCAAAG	GAGCGGATCG	780
GGATCGCAAT	CCTGACAGAG	ACTAGGGTTT	TTTGTTCCAA	TATGGAACGT	AAAAAATTAA	840
CCTGTGTTTC	ATATCAGAAC	AAAAAGGCGA	AAGATTTTTT	TGTTCCCTGC	CGGCCCTACA	900
GTGATCGCAC	TGCTCCGGTA	CGCTCCGTTT	AGGCCGCGCT	TCACTGGCCG	GCGCGGATAA	960
CGCCAGGGCT	CATCATGTCT	ACATGCGCAC	TTATTTGAGG	GTGAAAGGAA	TGCTAAAAGT	1020
TATTCAATCT	CCAGCCAAAT	ATCTTCAGGG	TCCTGATGCT	GCTGTTCTGT	TCGGTCAATA	1080
TGCCAAAAAC	CTGGCGGAGA	GCTTCTTCGT	CATCGCTGAC	GATTTCGTAA	TGAAGCTGGC	1140
GGGAGAGAAA	GTGGTGAATG	GCCTGCAGAG	CCACGATATT	CGCTGCCATG	CGGAACGGTT	1200
TAACGGCGAA	TGCAGCCATG	CGGAAATCAA	CCGTCTGATG	GCGATTTTGC	AAAAACAGGG	1260
CTGCCGCGGC	GTGGTCGGGA	TCGGCGGTGG	TAAAACCCTC	GATACCGCGA	AGGCGATCGG	1320
TTACTACCAG	AAGCTGCCGG	TGGTGGTGAT	CCCACCATC	GCCTCGACCG	ATGCGCCAAC	1380
CAGCGCGCTG	TCGGTGATCT	ACACCGAAGC	GGGCGAGTTT	GAAGAGTATC	TGATCTATCC	1440
GAAAAACCCG	GATATGGTGG	TGATGGACAC	GGCGATTATC	GCCAAAGCGC	CGGTACGCCT	1500

GCTGGTCTCC GGCATGGGCG ATGCGCTCTC CACCTGGTTC GAGGCCAAAG CTTGCTACGA 1560
TGCGCGCGCC ACCAGCATGG CCGGAGGACA GTCCACCGAG GCGGCGCTGA GCCTCGCCCCG 1620
CCTGTGCTAT GATACGCTGC TGGCGGAGGG CGAAAAGGCC CGTCTGGCGG CGCAGGCCGG 1680
GGTAGTGACC GAAGCGCTGG AGCGCATCAT CGAGGCGAAC ACTTACCTCA GCGGCATTGG 1740
CTTTGAAAGC AGTGGCCTGG CCGCTGCCCCA TGCAATCCAC AACGGTTTCA CCATTCTTGA 1800
AGAGTGCCAT CACCTGTATC ACGGTGAGAA AGTGGCCTTC GGTACCCTGG CGCAGCTGGT 1860
GCTGCAGAAC AGCCCGATGG ACGAGATTGA AACGGTGCAG GGCTTCTGCC AGCGCGTCGG 1920
CCTGCCGGTG ACGCTCGCGC AGATGGGCGT CAAAGAGGGG ATCGACGAGA AAATCGCCGC 1980
GGTGGCGAAA GCTACCTGCG CGGAAGGGGA AACCATCCAT AATATGCCGT TTGCGGTGAC 2040
CCCGGAGAGC GTCCATGCCG CTATCCTCAC CGCCGATCTG TTAGGCCAGC AGTGGCTGGC 2100
GCGTTAATTC GCGGTGGCTA AACCGCTGGC CCAGGTCAGC GGTTTTTCTT TCTCCCCTCC 2160
GGCAGTCGCT GCCGGAGGGG TTCTCTATGG TACAACGCGG AAAAGGATAT GACTGTTTCA 2220
ACTCAGGATA CCGGGAAGGC GGTCTCTTCC GTCATTGCCC AGTCATGGCA CCGCTGCAGC 2280
AAGTTTATGC AGCGCGAAAC CTGGCAAACG CCGCACCAGG CCCAGGGCCT GACCTTCGAC 2340
TCCATCTGTC GGCCTAAAC CGCGCTGCTC ACCATCGGCC AGGCGGCGCT GGAAGACGCC 2400
TGGGAGTTTA TGGACGGCCG CCCCTGCGCG CTGTTTATTC TTGATGAGTC CGCCTGCATC 2460
CTGAGCCGTT GCGGCGAGCC GCAAACCCTG GCCCAGCTGG CTGCCCTGGG ATTTTCGCGAC 2520
GGCAGCTATT GTGCGGAGAG CATTATCGGC ACCTGCGCGC TGTCGCTGGC CGCGATGCAG 2580
GGCCAGCCGA TCAACACCGC CGGCGATCGG CATTTTAAGC AGGCGCTACA GCCATGGAGT 2640
TTTTGCTCGA CGCCGGTGTT TGATAACCAC GGGCGGCTGT TCGGCTCTAT CTCGCTTTGC 2700
TGTCTGGTCG AGCACCAGTC CAGCGCCGAC CTCTCCCTGA CGCTGGCCAT CGCCCGCGAG 2760
GTGGGTA ACT CCCTGCTTAC CGACAGCCTG CTGGCGGAAT CCAACCGTCA CCTCAATCAG 2820
ATGTACGGCC TGCTGGAGAG CATGGACGAT GGGGTGATGG CGTGGAACGA ACAGGGCGTG 2880
CTGCAGTTTC TCAATGTTCA GCGGCGGAGA CTGCTGCATC TTGATGCTCA GGCCAGCCAG 2940
GGGAAAATA TCGCCGATCT GGTGACCCTC CCGGCGCTGC TGCGCCGCGC CATCAAACAC 3000
GCCCCGCGCC TGAATCACGT CGAAGTCACC TTTGAAAGTC AGCATCAGTT TGTCGATGCG 3060
GTGATCACCT TAAAACCGAT TGTCGAGGCG CAAGGCAACA GTTTTATTCT GCTGCTGCAT 3120
CCGGTGGAGC AGATGCGGCA GCTGATGACC AGCCAGCTCG GTAAAGTCAG CCACACCTTT 3180

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GAGCAGATGT	CTGCCGACGA	TCCGGAAACC	CGACGCCTGA	TCCACTTTGG	CCGCCAGGCG	3240
GCGCGCGGCG	GCTTCCCGGT	GCTACTGTGC	GGCGAAGAGG	GGGTCGGGAA	AGAGCTGCTG	3300
AGCCAGGCTA	TTCACAATGA	AAGCGAACGG	GCGGGCGGCC	CCTACATCTC	CGTCAACTGC	3360
CAGCTATATG	CCGACAGCGT	GCTGGGCCAG	GACTTTATGG	GCAGCGCCCC	TACCGACGAT	3420
GAAAATGGTC	GCCTGAGCCG	CCTTGAGCTG	GCCAACGGCG	GCACCCTGTT	TCTGGAAAAG	3480
ATCGAGTATC	TGGCGCCGGA	GCTGCAGTCG	GCTCTGCTGC	AGGTGATTAA	GCAGGGCGTG	3540
CTCACCCGCC	TCGACGCCCC	GCGCCTGATC	CCGGTGGATG	TGAAGGTGAT	TGCCACCACC	3600
ACCGTCGATC	TGGCCAATCT	GGTGGAACAG	AACCGCTTTA	GCCGCCAGCT	GTAATATGCG	3660
CTGCACTCCT	TTGAGATCGT	CATCCCGCCG	CTGCGCGCCC	GACGCAACAG	TATTCCGTCG	3720
CTGGTGCATA	ACCGGTTGAA	GAGCCTGGAG	AAGCGTTTCT	CTTCGCGACT	GAAAGTGGAC	3780
GATGACGCGC	TGGCACAGCT	GGTGGCCTAC	TCGTGGCCGG	GGAATGATTT	TGAGCTCAAC	3840
AGCGTCATTG	AGAATATCGC	CATCAGCAGC	GACAACGGCC	ACATTGCGCT	GAGTAATCTG	3900
CCGGAATATC	TCTTTTCCGA	GCGGCCGGGC	GGGGATAGCG	CGTCATCGCT	GCTGCCGGCC	3960
AGCCTGACTT	TTAGCGCCAT	CGAAAAGGAA	GCTATTATTC	ACGCCGCCCG	GGTGACCAGC	4020
GGGCGGGTGC	AGGAGATGTC	GCAGCTGCTC	AATATCGGCC	GCACCACCCT	GTGGCGCAAA	4080
ATGAAGCAGT	ACGATATTGA	CGCCAGCCAG	TTCAAGCGCA	AGCATCAGGC	CTAGTCTCTT	4140
CGATTGCGGC	CATGGAGAAC	AGGGCATCCG	ACAGGCGATT	GCTGTAGCGT	TTGAGCGCGT	4200
CGCGCAGCGG	ATGCGCGCGG	TCCATGGCCG	TCAGCAGGCG	TTCGAGCCGA	CGGGACTGGG	4260
TGCGCGCCAC	GTGCAGCTGG	GCAGAGGCGA	GATTCCCTCC	CGGGATCACG	AACTGTTTTA	4320
ACGGGCCGCT	CTCGGCCATA	TTGCGGTCGA	TAAGCCGCTC	CAGGGCGGTG	ATCTCCTCTT	4380
CGCCGATCGT	CTGGCTCAGG	CGGGTCAGGC	CCCGCGCATC	GCTGGCCAGT	TCAGCCCCCA	4440
GCACGAACAG	CGTCTGCTGA	ATATGGTGCA	GGCTTTCCCG	CAGCCCGGCG	TCGCGGGTCG	4500
TGGCGTAGCA	GACGCCCAGC	TGGGATATCA	GTTTCATCGAC	GGTGCCGTAG	GCCTCGACGC	4560
GAATATGGTC	TTTCTCGATG	CGGCTGCCGC	CGTACAGGGC	GGTGGTGCCT	TTATCCCCGG	4620
TGCGGGTATA	GATACGATAC	ATTCACTTTC	TCTCACTTAA	CGGCAGGACT	TTAACCAGCT	4680
GCCCGGCGTT	GGCGCCGAGC	GTACGCAGTT	GATCGTCGCT	ATCGGTGACG	TGTCCGGTAG	4740
CCAGCGGCGC	GTCCGCCGGC	AGCTGGGCAT	GAGTGAGGGC	TATCTCGCCG	GACGCGCTGA	4800
GCCCGATAAC	CACCCGCAGG	GGCGAGCTTC	TGGCCGCCAG	GGCGCCAGC	GCAGCGGCGT	4860

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CACCGCCTCC	GTCATAGGTT	ATGGTCTGGC	AGGGGACCCC	CTGCTCCTCC	AGCCCCCAGC	4920
ACAGCTCATT	GATGGCGCCG	GCATGGTGCC	CGCGCGGATC	GTAAAACAGG	CGTACGCCGTG	4980
GCGGTGAAAG	CGACATGACG	GTCCCCCTCGT	TAACACTCAG	AATGCCTGGC	GGAAAATCGC	5040
GGCAATCTCC	TGCTCGTTGC	CTTTACGCGG	GTTTCGAGAAC	GCATTGCCGT	CTTTTAGAGC	5100
CATCTCCGCC	ATGTAGGGGA	AGTCGGCCTC	TTTTACCCCC	AGATCGCGCA	GATGCTGCGG	5160
AATACCGATA	TCCATCGACA	GACGCGTGAT	AGCGGCGATG	GCTTTTTCCG	CCGCGTCGAG	5220
AGTGGACAGT	CCGGTGATAT	TTTCGCCCCAT	CAGTTCAGCG	ATATCGGCGA	ATTTCTCCGG	5280
GTTGGCGATC	AGGTTGTAGC	GCGCCACATG	CGGCAGCAGG	ACAGCGTTGG	CCACGCCGTG	5340
CGGCATGTCT	TACAGGCCGC	CCAGCTGGTG	CGCCATGGCG	TGCACGTAGC	CGAGGTTGGC	5400
GTTATTGAAA	GCCATCCCCG	CCAGCAGAGA	AGCATAGGCC	ATGTTTTCCC	GCGCCTGCAG	5460
ATTGCTGCCG	AGGGCCACGG	CCTGGCGCAG	GTTGCGGGCG	ATGAGGCGGA	TCGCCTGCAT	5520
GGCGGCGGCG	TCCGTCACCG	GGTTAGCGTC	TTTGGAGATA	TAGGCCTCTA	CGGCGTGGGT	5580
CAGGGCATCC	ATCCCGGTCT	CCGCGGTCAG	GGCGGCCGGT	TTACCGATCA	TCAGCAGTGG	5640
ATCGTTGATA	GAGACCGACG	GCAGTTTGCG	CCAGCTGACG	ATCACAACT	TCACTTTGGT	5700
TTCGGTGTTG	GTCAGGACGC	AGTGGCGGGT	GACCTCGCTG	GCGGTGCCGG	CGGTGGTATT	5760
GACCGCGACG	ATAGGCGGCA	GCGGGTTGGT	CAGGGTCTCG	ATTCCGGCAT	ACTGGTACAG	5820
ATCGCCCTCA	TGGGTGGCGG	CGATGCCGAT	GCCTTTGCCG	CAATCGTGCG	GGCTGCCGCC	5880
GCCCACGGTG	ACGATGATGT	CGCACTGTTC	GCGGCGAAAC	ACGGCGAGGC	CGTCGCGCAC	5940
GTTGGTGTCT	TTCGGGTTCG	GCTCGACGCC	GTCAAAGATC	GCCACCTCGA	TCCCGGCCTC	6000
CCGCAGATAA	TGCAGGGTTT	TGTCCACCGC	GCCATCTTTA	ATTGCCCGCA	GGCCTTTGTC	6060
GGTGACCAGC	AGGGCTTTTT	TCCCCCCCAG	CAGCTGGCAG	CGTTCGCCGA	CTACGGAAAT	6120
GGCGTTGGGG	CCAAAAAAGT	TAACGTTTGG	CACCAGATAA	TCAAACATAC	GATAGCTCAT	6180
AATATACCTT	CTCGCTTCAG	GTTATAATGC	GGAAAAACAA	TCCAGGGCGC	ACTGGGCTAA	6240
TAATTGATCC	TGCTCGACCG	TACCGCCGCT	AACGCCGACG	GCGCCAATTA	CCTGCTCAT	6300
AAAAATAACT	GGCAGGCCGC	CGCCAAAAAT	AATAATTCGC	TGTTGGTTGG	TTAGCTGCAG	6360
ACCGTACAGA	GATTGTCCTG	GCTGGACCGC	TGACGTAATT	TCATGGGTAC	CTTGCTTCAG	6420
GCTGCAGGCG	CTCCAGGCTT	TATTCAGGGA	AATATCGCAG	CTGGAGACGA	AGGCCTCGTC	6480
CATCCGCTGG	ATAAGCAGCG	TGTTGCCTCC	GCGGTCAACT	ACGGAAAACA	CCACCGCCAC	6540

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GTTGATCTCA	GTGGCTTTTT	TTTCCACCGC	CGCCGCCATT	TGCTGGGCGG	CGGCCAGGGT	6600
GATTGTCTGA	ACTTGTTGGC	TCTTGTTTAT	CATTCTCTCC	CGCACCAGGA	TAACGCTGGC	6660
GCGAATAGTC	AGTAGGGGGC	GATAGTAAAA	AACTATTACC	ATTCGGTTGG	CTTGCTTTAT	6720
TTTTGTCAGC	GTTATTTTGT	CGCCCGCCAT	GATTTAGTCA	ATAGGGTTAA	AATAGCGTCG	6780
GAAAAACGTA	ATTAAGGGCG	TTTTTTTATTA	ATTGATTTAT	ATCATTGCGG	GCGATCACAT	6840
TTTTTATTTT	TGCCGCCGGA	GTAAAGTTTC	ATAGTGAAAC	TGTCGGTAGA	TTTCGTGTGC	6900
CAAATTGAAA	CGAAATTAAA	TTTATTTTTT	TCACCACTGG	CTCATTTAAA	GTTCCGCTAT	6960
TGCCGGTAAT	GGCCGGGCGG	CAACGACGCT	GGCCCGGCGT	ATTCGCTACC	GTCTGCGGAT	7020
TTCACCTTTT	GAGCCGATGA	ACAATGAAAA	GATCAAAACG	ATTTGCAGTA	CTGGCCCAGC	7080
GCCCCGTCAA	TCAGGACGGG	CTGATTGGCG	AGTGGCCTGA	AGAGGGGCTG	ATCGCCATGG	7140
ACAGCCCCTT	TGACCCGGTC	TCTTCAGTAA	AAGTGGACAA	CGGTCTGATC	GTCGAACTGG	7200
ACGGCAAACG	CCGGGACCAG	TTTGACATGA	TCGACCGATT	TATCGCCGAT	TACGCGATCA	7260
ACGTTGAGCG	CACAGAGCAG	GCAATGCGCC	TGGAGGCGGT	GGAAATAGCC	CGTATGCTGG	7320
TGGATATTCA	CGTCAGCCGG	GAGGAGATCA	TTGCCATCAC	TACCGCCATC	ACGCCGGCCA	7380
AAGCGGTCGA	GGTGATGGCG	CAGATGAACG	TGGTGGAGAT	GATGATGGCG	CTGCAGAAGA	7440
TGCGTGCCCG	CCGGACCCCC	TCCAACCACT	GCCACGTCAC	CAATCTCAAA	GATAATCCGG	7500
TGCAGATTGC	CGCTGACGCC	GCCGAGGCCG	GGATCCGCGG	CTTCTCAGAA	CAGGAGACCA	7560
CGGTGCGTAT	CGCGCGCTAC	GCGCCGTTTA	ACGCCCTGGC	GCTGTTGGTC	GGTTCGCAGT	7620
GCGGCCGCCC	CGGCGTGTTG	ACGCAGTGCT	CGGTGGAAGA	GGCCACCGAG	CTGGAGCTGG	7680
GCATGCGTGG	CTTAACCAGC	TACGCCGAGA	CGGTGTCGGT	CTACGGCACC	GAAGCGGTAT	7740
TTACCGACGG	CGATGATACG	CCGTGGTCAA	AGGCGTTCCT	CGCCTCGGCC	TACGCCTCCC	7800
GCGGGTTGAA	AATGCGCTAC	ACCTCCGGCA	CCGGATCCGA	AGCGCTGATG	GGCTATTCGG	7860
AGAGCAAGTC	GATGCTCTAC	CTCGAATCGC	GCTGCATCTT	CATTACTAAA	GGCGCCGGGG	7920
TTCAGGGACT	GCAAAACGGC	GCGGTGAGCT	GTATCGGCAT	GACCGGCGCT	GTGCCGTCGG	7980
GCATTCGGGC	GGTGCTGGCG	GAAAACCTGA	TCGCCTCTAT	GCTCGACCTC	GAAGTGGCGT	8040
CCGCCAACGA	CCAGACTTTC	TCCCACTCGG	ATATTCGCCG	CACCGCGCGC	ACCCTGATGC	8100
AGATGCTGCC	GGGCACCGAC	TTTATTTTCT	CCGGCTACAG	CGCGGTGCCG	AACTACGACA	8160
ACATGTTGCG	CGGCTCGAAC	TTCGATGCGG	AAGATTTTGA	TGATTACAAC	ATCCTGCAGC	8220

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GTGACCTGAT GGTTGACGGC GGCCTGCGTC CGGTGACCGA GGCGGAAACC ATTGCCATTC	8280
GCCAGAAAGC GGCGCGGGCG ATCCAGGCGG TTTTCCGCGA GCTGGGGCTG CCGCCAATCG	8340
CCGACGAGGA GGTGGAGGCC GCCACCTACG CGCACGGCAG CAACGAGATG CCGCCGCGTA	8400
ACGTGGTGGA GGATCTGAGT GCGGTGGAAG AGATGATGAA GCGCAACATC ACCGGCCTCG	8460
ATATTGTCGG CGCGCTGAGC CGCAGCGGCT TTGAGGATAT CGCCAGCAAT ATTCTCAATA	8520
TGCTGCGCCA GCGGGTCACC GGCGATTACC TGCAGACCTC GGCCATTCTC GATCGGCAGT	8580
TCGAGGTGGT GAGTGCGGTC AACGACATCA ATGACTATCA GGGGCCGGGC ACCGGCTATC	8640
GCATCTCTGC CGAACGCTGG GCGGAGATCA AAAATATTCC GGGCGTGGTT CAGCCCGACA	8700
CCATTGAATA AGGCGGTATT CCTGTGCAAC AGACAACCCA AATTCAGCCC TCTTTTACCC	8760
TGAAAACCCG CGAGGGCGGG GTAGCTTCTG CCGATGAACG CGCCGATGAA GTGGTGATCG	8820
GCGTCGGCCC TGCCTTCGAT AAACACCAGC ATCACACTCT GATCGATATG CCCCATGGCG	8880
CGATCCTCAA AGAGCTGATT GCCGGGGTGG AAGAAGAGGG GCTTCACGCC CGGGTGGTGC	8940
GCATTCTGCG CACGTCCGAC GTCTCCTTTA TGGCCTGGGA TCGGCGCAAC CTGAGCGGCT	9000
CGGGGATCGG CATCGGTATC CAGTCGAAGG GGACCACGGT CATCCATCAG CGCGATCTGC	9060
TGCCGCTCAG CAACCTGGAG CTGTTCTCCC AGGCGCCGCT GCTGACGCTG GAGACCTACC	9120
GGCAGATTGG CAAAAACGCT GCGCGCTATG CGCGCAAAGA GTCACCTTCG CCGGTGCCGG	9180
TGGTGAACGA TCAGATGGTG CGGCCGAAAT TTATGGCCAA AGCCGCGCTA TTTCATATCA	9240
AAGAGACCAA ACATGTGGTG CAGGACGCCG AGCCCGTCAC CCTGCACATC GACTTAGTAA	9300
GGGAGTGACC ATGAGCGAGA AAACCATGCG CGTGCAGGAT TATCCGTTAG CCACCCGCTG	9360
CCCGGAGCAT ATCCTGACGC CTACCGGCAA ACCATTGACC GATATTACCC TCGAGAAGGT	9420
GCTCTCTGGC GAGGTGGGCC CGCAGGATGT GCGGATCTCC CGCCAGACCC TTGAGTACCA	9480
GGCGCAGATT GCCGAGCAGA TGCAGCGCCA TGCGGTGGCG CGCAATTTCC GCCGCGCGGC	9540
GGAGCTTATC GCCATTCTTG ACGAGCGCAT TCTGGCTATC TATAACGCGC TCGCCCCGTT	9600
CCGCTCCTCG CAGGCGGAGC TGCTGGCGAT CGCCGACGAG CTGGAGCACA CCTGGCATGC	9660
GACAGTGAAT GCCGCCTTTG TCCGGGAGTC GGCGGAAGTG TATCAGCAGC GGCATAAGCT	9720
GCGTAAAGGA AGCTAAGCGG AGGTCAGCAT GCCGTTAATA GCCGGGATTG ATATCGGCAA	9780
CGCCACCACC GAGGTGGCGC TGGCGTCCGA CTACCCGCAG GCGAGGGCGT TTGTTGCCAG	9840
CGGGATCGTC GCGACGACGG GCATGAAAGG GACGCGGGAC AATATCGCCG GGACCCCTCGC	9900

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CGCGCTGGAG CAGGCCCTGG CGAAAACACC GTGGTCGATG AGCGATGTCT CTCGCATCTA 9960
TCTTAACGAA GCCGCGCCGG TGATTGGCGA TGTGGCGATG GAGACCATCA CCGAGACCAT 10020
TATCACCGAA TCGACCATGA TCGGTCATAA CCCGCAGACG CCGGGCGGGG TGGGCGTTGG 10080
CGTGGGGACG ACTATCGCCC TCGGGCGGCT GCGACGCTG CCGGCGGCGC AGTATGCCGA 10140
GGGGTGGATC GTACTGATTG ACGACGCCGT CGATTTCCCTT GACGCCGTGT GGTGGCTCAA 10200
TGAGGCGCTC GACCGGGGGA TCAACGTGGT GCGGCGATC CTCAAAAGG ACGACGGCGT 10260
GCTGGTGAAC AACCGCCTGC GTAAAACCTT GCCGGTGGTG GATGAAGTGA CGCTGCTGGA 10320
GCAGGTCCCC GAGGGGGTAA TGGCGGCGGT GGAAGTGGCC GCGCCGGGCC AGGTGGTGCG 10380
GATCCTGTCG AATCCCTACG GGATCGCCAC CTTCTTCGGG CTAAGCCCGG AAGAGACCCA 10440
GGCCATCGTC CCCATCGCCC GCGCCCTGAT TGGCAACCGT TCCGCGGTGG TGCTCAAGAC 10500
CCCGCAGGGG GATGTGCACT CGCGGGTGAT CCCGGCGGGC AACCTCTACA TTAGCGGCGA 10560
AAAGCGCCGC GGAGAGGCCG ATGTCGCCGA GGGCGCGGAA GCCATCATGC AGGCGATGAG 10620
CGCCTGCGCT CCGGTACGCG ACATCCGCGG CGAACCAGGC ACCCACGCCG GCGGCATGCT 10680
TGAGCGGGTG CGCAAGGTAA TGGCGTCCCT GACCGGCCAT GAGATGAGCG CGATATACAT 10740
CCAGGATCTG CTGGCGGTGG ATACGTTTAT TCCGCGCAAG GTGCAGGGCG GGATGGCCGG 10800
CGAGTGCGCC ATGGAGAATG CCGTCGGGAT GCGGCGGATG GTGAAAGCGG ATCGTCTGCA 10860
AATGCAGGTT ATCGCCCGCG AACTGAGCGC CCGACTGCAG ACCGAGGTGG TGGTGGGCGG 10920
CGTGGAGGCC AACATGGCCA TCGCCGGGGC GTTAACCACT CCCGGCTGTG CGGCGCCGCT 10980
GGCGATCCTC GACCTCGGCG CCGGCTCGAC GGATGCGGCG ATCGTCAACG CGGAGGGGCA 11040
GATAACGGCG GTCCATCTCG CCGGGGCGGG GAATATGGTC AGCCTGTTGA TTAAAACCGA 11100
GCTGGGCCTC GAGGATCTTT CGCTGGCGGA AGCGATAAAA AAATACCCGC TGGCCAAAGT 11160
GGAAAGCCTG TTCAGTATTC GTCACGAGAA TGGCGCGGTG GAGTTCTTTC GGAAGCCCT 11220
CAGCCCGGCG GTGTTGCCA AAGTGGTGTA CATCAAGGAG GGCGAACTGG TGCCGATCGA 11280
TAACGCCAGC CCGCTGGAAA AAATTCGTCT CGTGCGCCGG CAGGCGAAAG AGAAAGTGTT 11340
TGTCACCAAC TGCCTGCGCG CGCTGCGCCA GGTCTCACCC GCGGTTCCA TTCGCGATAT 11400
CGCCTTTGTG GTGCTGGTGG GCGGCTCATC GCTGGACTTT GAGATCCCGC AGCTTATCAC 11460
GGAAGCCTTG TCGCACTATG GCGTGGTCGC CGGGCAGGGC AATATTCGGG GAACAGAAGG 11520
GCCGCGCAAT GCGGTGCGCA CCGGGCTGCT ACTGGCCGGT CAGGCGAATT AAACGGGCGC 11580

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TCGCGCCAGC CTCTCTCTTT AACGTGCTAT TTCAGGATGC CGATAATGAA CCAGACTTCT 11640

ACCTTAACCG GGCAGTGCCT GGCCGAGTTT CTTGGCACCG GATTGCTCAT TTCTTCGGC 11700

GCGGGCTGCG TCGCTGCGCT GCGGGTCGCC GGGGCCAGCT TTGGTCAGTG GGAGATCAGT 11760

ATTATCTGGG GCTTGGCGT CGCCATGGCC ATCTACCTGA CGGCCGGTGT CTCCGGCGCG 11820

CACCTAAATC CGGCGGTGAC CATTGCCCTG TGGCTGTTCT CTTGTTTTGA ACGCCGCAAG 11880

GTGCTGCCGT TTATTGTTGC CCAGACGGCC GGGGCCCTTCT GCGCCGCCGC GCTGGTGTAT 11940

GGGCTCTATC GCCAGCTGTT TCTCGATCTT GAACAGAGTC AGCATATCGT GCGCGGCACT 12000

GCGCCAGTC TTAACCTGGC CGGGGTCTTT TCCACGTACC CGCATCCACA TATCACTTTT 12060

ATAACAAGCGT TTGCCGTGGA GACCACCATC ACGGCAATCC TGATGGCGAT GATCATGGCC 12120

CTGACCGACG ACGGCAACGG AATTC 12145

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 94 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGCTTAGGAG TCTAGAATAT TGAGCTCGAA TTCCGGGGCA TGCGGTACCG GATCCAGAAA 60

AAAGCCCGCA CCTGACAGTG CGGGCTTTTT TTTT 94

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGAATTCAGA TCTCAGCAAT GAGCGAGAAA ACCATGC

37

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid

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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCTCTAGATT AGCTTCCTTT ACGCAGC

27

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGCCAAGCTT AAGGAGGTTA ATTAAATGAA AAG

33

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GCTCTAGATT ATTCAATGGT GTCGGG

26

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 42 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GCGCCGTCTA GAATTATGAG CTATCGTATG TTTGATTATC TG

42

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid

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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TTTCTGATACGG GATCCTCAGA ATGCCTGGCG GAAAT

36

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 51 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TTTTCGCGCGATCC AGGAGTCTAG AATTATGGGA TTGACTACTA AACCTCTATC T

51

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TTT GATACGCCCG GGTACCATT TCAACAGATC GTCCTT

36

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TCGACGAATT CAGGAGGA

18

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid

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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CTAGTCCTCC TGAATTCG

18

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CTAGTAAGGA GGACAATTC

19

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TCATGGAATTG TCCTCCTTA

19

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 271 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: GPPI

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Met Lys Arg Phe Asn Val Leu Lys Tyr Ile Arg Thr Thr Lys Ala Asn
1 5 10 15

Ile Gln Thr Ile Ala Met Pro Leu Thr Thr Lys Pro Leu Ser Leu Lys

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20	25	30
Ile Asn Ala Ala Leu Phe Asp Val Asp Gly Thr Ile Ile Ile Ser Gln		
35	40	45
Pro Ala Ile Ala Ala Phe Trp Arg Asp Phe Gly Lys Asp Lys Pro Tyr		
50	55	60
Phe Asp Ala Glu His Val Ile His Ile Ser His Gly Trp Arg Thr Tyr		
65	70	75
Asp Ala Ile Ala Lys Phe Ala Pro Asp Phe Ala Asp Glu Glu Tyr Val		
85	90	95
Asn Lys Leu Glu Gly Glu Ile Pro Glu Lys Tyr Gly Glu His Ser Ile		
100	105	110
Glu Val Pro Gly Ala Val Lys Leu Cys Asn Ala Leu Asn Ala Leu Pro		
115	120	125
Lys Glu Lys Trp Ala Val Ala Thr Ser Gly Thr Arg Asp Met Ala Lys		
130	135	140
Lys Trp Phe Asp Ile Leu Lys Ile Lys Arg Pro Glu Tyr Phe Ile Thr		
145	150	155
Ala Asn Asp Val Lys Gln Gly Lys Pro His Pro Glu Pro Tyr Leu Lys		
165	170	175
Gly Arg Asn Gly Leu Gly Phe Pro Ile Asn Glu Gln Asp Pro Ser Lys		
180	185	190
Ser Lys Val Val Val Phe Glu Asp Ala Pro Ala Gly Ile Ala Ala Gly		
195	200	205
Lys Ala Ala Gly Cys Lys Ile Val Gly Ile Ala Thr Thr Phe Asp Leu		
210	215	220
Asp Phe Leu Lys Glu Lys Gly Cys Asp Ile Ile Val Lys Asn His Glu		
225	230	235
Ser Ile Arg Val Gly Glu Tyr Asn Ala Glu Thr Asp Glu Val Glu Leu		
245	250	255
Ile Phe Asp Asp Tyr Leu Tyr Ala Lys Asp Asp Leu Leu Lys Trp		
260	265	270

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 555 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

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(vi) ORIGINAL SOURCE:

(A) ORGANISM: DHAB1

(xi) -- SEQUENCE DESCRIPTION: -- SEQ. ID NO:34:

Met Lys Arg Ser Lys Arg Phe Ala Val Leu Ala Gln Arg Pro Val Asn
 1 5 10 15

Gln Asp Gly Leu Ile Gly Glu Trp Pro Glu Glu Gly Leu Ile Ala Met
 20 25 30

Asp Ser Pro Phe Asp Pro Val Ser Ser Val Lys Val Asp Asn Gly Leu
 35 40 45

Ile Val Glu Leu Asp Gly Lys Arg Arg Asp Gln Phe Asp Met Ile Asp
 50 55 60

Arg Phe Ile Ala Asp Tyr Ala Ile Asn Val Glu Arg Thr Glu Gln Ala
 65 70 75 80

Met Arg Leu Glu Ala Val Glu Ile Ala Arg Met Leu Val Asp Ile His
 85 90 95

Val Ser Arg Glu Glu Ile Ile Ala Ile Thr Thr Ala Ile Thr Pro Ala
 100 105 110

Lys Ala Val Glu Val Met Ala Gln Met Asn Val Val Glu Met Met Met
 115 120 125

Ala Leu Gln Lys Met Arg Ala Arg Arg Thr Pro Ser Asn Gln Cys His
 130 135 140

Val Thr Asn Leu Lys Asp Asn Pro Val Gln Ile Ala Ala Asp Ala Ala
 145 150 155 160

Glu Ala Gly Ile Arg Gly Phe Ser Glu Gln Glu Thr Thr Val Gly Ile
 165 170 175

Ala Arg Tyr Ala Pro Phe Asn Ala Leu Ala Leu Leu Val Gly Ser Gln
 180 185 190

Cys Gly Arg Pro Gly Val Leu Thr Gln Cys Ser Val Glu Glu Ala Thr
 195 200 205

Glu Leu Glu Leu Gly Met Arg Gly Leu Thr Ser Tyr Ala Glu Thr Val
 210 215 220

Ser Val Tyr Gly Thr Glu Ala Val Phe Thr Asp Gly Asp Asp Thr Pro
 225 230 235 240

Trp Ser Lys Ala Phe Leu Ala Ser Ala Tyr Ala Ser Arg Gly Leu Lys
 245 250 255

Met Arg Tyr Thr Ser Gly Thr Gly Ser Glu Ala Leu Met Gly Tyr Ser
 260 265 270

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Glu Ser Lys Ser Met Leu Tyr Leu Glu Ser Arg Cys Ile Phe Ile Thr
 275 280 285

Lys Gly Ala Gly Val Gln Gly Leu Gln Asn Gly Ala Val Ser Cys Ile
 290 295 300

Gly Met Thr Gly Ala Val Pro Ser Gly Ile Arg Ala Val Leu Ala Glu
 305 310 315 320

Asn Leu Ile Ala Ser Met Leu Asp Leu Glu Val Ala Ser Ala Asn Asp
 325 330 335

Gln Thr Phe Ser His Ser Asp Ile Arg Arg Thr Ala Arg Thr Leu Met
 340 345 350

Gln Met Leu Pro Gly Thr Asp Phe Ile Phe Ser Gly Tyr Ser Ala Val
 355 360 365

Pro Asn Tyr Asp Asn Met Phe Ala Gly Ser Asn Phe Asp Ala Glu Asp
 370 375 380

Phe Asp Asp Tyr Asn Ile Leu Gln Arg Asp Leu Met Val Asp Gly Gly
 385 390 395 400

Leu Arg Pro Val Thr Glu Ala Glu Thr Ile Ala Ile Arg Gln Lys Ala
 405 410 415

Ala Arg Ala Ile Gln Ala Val Phe Arg Glu Leu Gly Leu Pro Pro Ile
 420 425 430

Ala Asp Glu Glu Val Glu Ala Ala Thr Tyr Ala His Gly Ser Asn Glu
 435 440 445

Met Pro Pro Arg Asn Val Val Glu Asp Leu Ser Ala Val Glu Glu Met
 450 455 460

Met Lys Arg Asn Ile Thr Gly Leu Asp Ile Val Gly Ala Leu Ser Arg
 465 470 475 480

Ser Gly Phe Glu Asp Ile Ala Ser Asn Ile Leu Asn Met Leu Arg Gln
 485 490 495

Arg Val Thr Gly Asp Tyr Leu Gln Thr Ser Ala Ile Leu Asp Arg Gln
 500 505 510

Phe Glu Val Val Ser Ala Val Asn Asp Ile Asn Asp Tyr Gln Gly Pro
 515 520 525

Gly Thr Gly Tyr Arg Ile Ser Ala Glu Arg Trp Ala Glu Ile Lys Asn
 530 535 540

Ile Pro Gly Val Val Gln Pro Asp Thr Ile Glu
 545 550 555

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(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 194 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

(A) ORGANISM: DHAB2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Met Gln Gln Thr Thr Gln Ile Gln Pro Ser Phe Thr Leu Lys Thr Arg
 1 5 10 15

Glu Gly Gly Val Ala Ser Ala Asp Glu Arg Ala Asp Glu Val Val Ile
 20 25 30

Gly Val Gly Pro Ala Phe Asp Lys His Gln His His Thr Leu Ile Asp
 35 40 45

Met Pro His Gly Ala Ile Leu Lys Glu Leu Ile Ala Gly Val Glu Glu
 50 55 60

Glu Gly Leu His Ala Arg Val Val Arg Ile Leu Arg Thr Ser Asp Val
 65 70 75 80

Ser Phe Met Ala Trp Asp Ala Ala Asn Leu Ser Gly Ser Gly Ile Gly
 85 90 95

Ile Gly Ile Gln Ser Lys Gly Thr Thr Val Ile His Gln Arg Asp Leu
 100 105 110

Leu Pro Leu Ser Asn Leu Glu Leu Phe Ser Gln Ala Pro Leu Leu Thr
 115 120 125

Leu Glu Thr Tyr Arg Gln Ile Gly Lys Asn Ala Ala Arg Tyr Ala Arg
 130 135 140

Lys Glu Ser Pro Ser Pro Val Pro Val Val Asn Asp Gln Met Val Arg
 145 150 155 160

Pro Lys Phe Met Ala Lys Ala Ala Leu Phe His Ile Lys Glu Thr Lys
 165 170 175

His Val Val Gln Asp Ala Glu Pro Val Thr Leu His Ile Asp Leu Val
 180 185 190

Arg Glu

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(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 140 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: DHAB3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

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Met Ser Glu Lys Thr Met Arg Val Gln Asp Tyr Pro Leu Ala Thr Arg
1      5      10      15
Cys Pro Glu His Ile Leu Thr Pro Thr Gly Lys Pro Leu Thr Asp Ile
20     25     30
Thr Leu Glu Lys Val Leu Ser Gly Glu Val Gly Pro Gln Asp Val Arg
35     40     45
Ile Ser Arg Gln Thr Leu Glu Tyr Gln Ala Gln Ile Ala Glu Gln Met
50     55     60
Gln His Ala Val Ala Arg Asn Phe Arg Arg Ala Ala Glu Leu Ile Ala
65     70     75     80
Ile Pro Asp Glu Arg Ile Leu Ala Ile Tyr Asn Ala Leu Arg Pro Phe
85     90     95
Arg Ser Ser Gln Ala Glu Leu Leu Ala Ile Ala Asp Glu Leu Glu His
100    105    110
Thr Trp His Ala Thr Val Asn Ala Ala Phe Val Arg Glu Ser Ala Glu
115    120    125
Val Tyr Gln Gln Arg His Lys Leu Arg Lys Gly Ser
130    135    140

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(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 387 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: DHAT

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Met Ser Tyr Arg Met Phe Asp Tyr Leu Val Pro Asn Val Asn Phe Phe
1 5 10 15

Gly Pro Asn Ala Ile Ser Val Val Gly Glu Arg Cys Gln Leu Leu Gly
20 25 30

Gly Lys Lys Ala Leu Leu Val Thr Asp Lys Gly Leu Arg Ala Ile Lys
35 40 45

Asp Gly Ala Val Asp Lys Thr Leu His Tyr Leu Arg Glu Ala Gly Ile
50 55 60

Glu Val Ala Ile Phe Asp Gly Val Glu Pro Asn Pro Lys Asp Thr Asn
65 70 75 80

Val Arg Asp Gly Leu Ala Val Phe Arg Arg Glu Gln Cys Asp Ile Ile
85 90 95

Val Thr Val Gly Gly Gly Ser Pro His Asp Cys Gly Lys Gly Ile Gly
100 105 110

Ile Ala Ala Thr His Glu Gly Asp Leu Tyr Gln Tyr Ala Gly Ile Glu
115 120 125

Thr Leu Thr Asn Pro Leu Pro Pro Ile Val Ala Val Asn Thr Thr Ala
130 135 140

Gly Thr Ala Ser Glu Val Thr Arg His Cys Val Leu Thr Asn Thr Glu
145 150 155 160

Thr Lys Val Lys Phe Val Ile Val Ser Trp Arg Lys Leu Pro Ser Val
165 170 175

Ser Ile Asn Asp Pro Leu Leu Met Ile Gly Lys Pro Ala Ala Leu Thr
180 185 190

Ala Ala Thr Gly Met Asp Ala Leu Thr His Ala Val Glu Ala Tyr Ile
195 200 205

Ser Lys Asp Ala Asn Pro Val Thr Asp Ala Ala Ala Met Gln Ala Ile
210 215 220

Arg Leu Ile Ala Arg Asn Leu Arg Gln Ala Val Ala Leu Gly Ser Asn
225 230 235 240

Leu Gln Ala Arg Glu Asn Met Ala Tyr Ala Ser Leu Leu Ala Gly Met
245 250 255

Ala Phe Asn Asn Ala Asn Leu Gly Tyr Val His Ala Met Ala His Gln
260 265 270

Leu Gly Gly Leu Tyr Asp Met Pro His Gly Val Ala Asn Ala Val Leu
275 280 285

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Leu Pro His Val Ala Arg Tyr Asn Leu Ile Ala Asn Pro Glu Lys Phe
 290 295 300

Ala Asp Ile Ala Glu Leu Met Gly Glu Asn Ile Thr Gly Leu Ser Thr
 305 310 315 320

Leu Asp Ala Ala Glu Lys Ala Ile Ala Ala Ile Thr Arg Leu Ser Met
 325 330 335

Asp Ile Gly Ile Pro Gln His Leu Arg Asp Leu Gly Val Lys Glu Ala
 340 345 350

Asp Phe Pro Tyr Met Ala Glu Met Ala Leu Lys Asp Gly Asn Ala Phe
 355 360 365

Ser Asn Pro Arg Lys Gly Asn Glu Gln Glu Ile Ala Ala Ile Phe Arg
 370 375 380

Gln Ala Phe
 385

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GCGAATTCAT GAGCTATCGT ATGTTTG

27

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 28 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GCGAATTCAG AATGCCTGGC GGAAAATC

28

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 28 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GGGAATTCAT GAGCGAGAAA ACCATGCG

28

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GCGAATTCTTAGCTTCCTTT ACGCAGC

27

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GCGAATTCAT GCAACAGACA ACCCAAATTC

30

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GCGAATTCAC TCCCTTACTA AGTCG

25

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GGGAATTCAT GAAAAGATCA AAACGATTG

30

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GGGAATTCCTTATTCAATGGT GTCGGGCTG

29

(2) INFORMATION FOR SEQ ID NO:46

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

TTGATAATAT AACCATGGCT GCTGCTGCTG ATAG

34

(2) INFORMATION FOR SEQ ID NO:47

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GTATGATATG TTATCTTGA TCCAATAAAT CTAATCTTC

39

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CATGACTAGT AAGGAGGACA ATTC

24

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CATGGAATTG TCCTCCTTAC TAGT

24

WHAT IS CLAIMED IS:

1. An improved method for the production of 1,3-propanediol from an organism capable of producing 1,3-propanediol, said organism comprising at least one gene encoding a dehydratase activity, the method comprising the steps of:
 - (a) introducing a gene encoding protein X into the organism to create a transformed organism; and
 - (b) culturing the transformed organism in the presence of at least one carbon source capable of being converted to 1,3 propanediol in said transformed host organism and under conditions suitable for the production of 1,3 propanediol wherein the carbon source is selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, and a one carbon substrate.
2. The method of Claim 1 further comprising the step of introducing at least one gene encoding a protein selected from the group consisting of protein 1, protein 2 and protein 3 into the organism.
3. The method of Claim 1 further comprising the step of recovering the 1,3 propanediol.
4. The method of Claim 1 wherein the gene encoding protein X is isolated from a glycerol dehydratase gene cluster.
5. The method of Claim 1 wherein the gene encoding protein X is isolated from a diol dehydratase gene cluster.
6. The method of Claim 4 wherein the glycerol dehydratase gene cluster is from an organism selected from the genera consisting of Klebsiella and Citrobactor.
7. The method of Claim 5 wherein the diol dehydratase gene cluster is from an organism selected from the genera consisting of Klebsiella, Clostridium and Salmonella.
8. The method of Claim 1 wherein the gene encoding a dehydratase activity is heterologous to the organism.
9. The method of Claim 1 wherein the gene encoding a dehydratase activity is homologous to the organism.

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10. The method of Claim 1 wherein the organism is selected from the group of genera consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*.

11. The method of Claim 10 wherein the organism is selected from the group consisting of *E.coli* and *Klebsiella spp.*

12. The method of Claim 1 wherein the gene encoding protein X is stably maintained in the host genome.

13. The method of Claim 2 wherein at least one gene encoding a protein selected from protein 1, protein 2 and protein 3 is stably maintained in the host genome.

14. The method of Claim 1 wherein the carbon source is glucose.

15. The method of Claim 1 wherein the gene encoding protein X has the sequence as shown in SEQ ID NO: 59.

16. The method of Claim 2 wherein protein 1 has the sequence as shown in SEQ ID NO: 60 or SEQ ID NO: 61.

17. The method of Claim 2 wherein protein 2 has the sequence as shown in SEQ ID NO: 62 or SEQ ID NO: 63.

18. The method of Claim 2 wherein protein 3 has the sequence as shown in SEQ ID NO: 64 or SEQ ID NO: 65.

19. A recombinant microorganism capable of producing 1,3-propanediol from a carbon source said recombinant microorganism comprising a) at least one gene encoding a dehydratase activity; b) at least one gene encoding a glycerol-3-phosphatase; and c) at least one gene encoding protein X.

20. The recombinant microorganism of Claim 19 further comprising d) at least one gene encoding a protein selected from the group consisting of protein 1, protein 2 and protein 3.

21. The recombinant microorganism of Claim 19 selected from the group consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*.

22. The recombinant microorganism of Claim 19 wherein the gene encoding protein X is isolated from a glycerol dehydratase gene cluster.

23. The recombinant microorganism of Claim 19 wherein the gene encoding protein X is isolated from a diol dehydratase gene cluster.

24. The recombinant microorganism of Claim 22 wherein the glycerol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella* and *Citrobacter*.

25. The recombinant microorganism of Claim 23 wherein the diol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella*, *Clostridium* and *Salmonella*.

26. The recombinant microorganism of Claim 19 wherein said dehydratase activity is heterologous to said microorganism.

27. The recombinant microorganism of Claim 19 wherein said dehydratase activity is homologous to said microorganism.

28. The recombinant microorganism of Claim 19 wherein the gene encoding protein X has the sequence as shown in SEQ ID NO: 59.

29. The recombinant microorganism of Claim 20 wherein protein 1 has the sequence as shown in SEQ ID NO: 60 or SEQ ID NO: 61.

30. The method of Claim 20 wherein protein 2 has the sequence as shown in SEQ ID NO: 62 or SEQ ID NO: 63.

31. The method of Claim 20 wherein protein 3 has the sequence as shown in SEQ ID: 64 or SEQ ID NO: 65.

32. A method for extending the half-life of dehydratase activity in a microorganism capable of producing 1,3-propanediol and containing at least one gene encoding a dehydratase activity, comprising the step of introducing a gene encoding protein X into said microorganism and culturing under conditions suitable for production of 1,3-propanediol.

33. The method of Claim 32 wherein the gene encoding the dehydratase activity is heterologous to said microorganism.

34. The method of Claim 32 wherein the gene encoding the dehydratase activity is homologous to said microorganism.

35. The microorganism of Claim 32 wherein the gene encoding protein X is isolated from a glycerol dehydratase gene cluster.

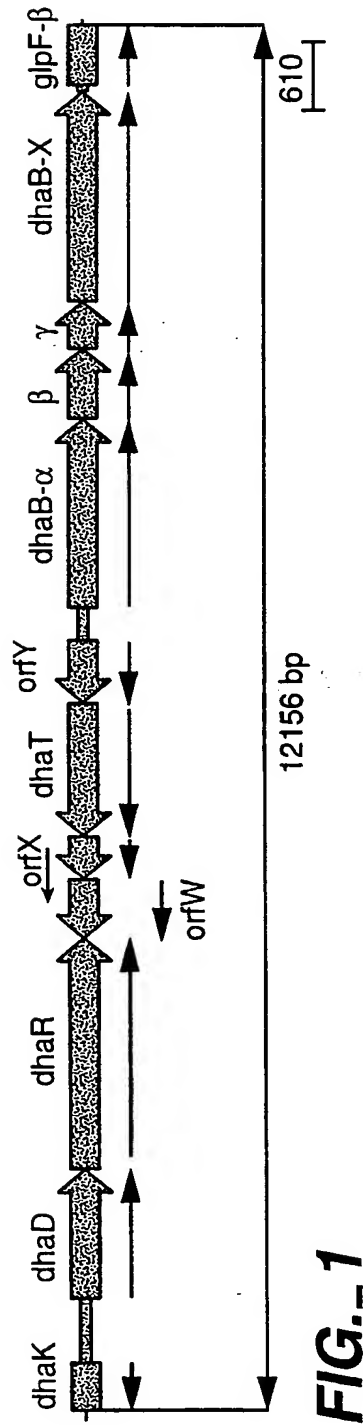
36. The microorganism of Claim 32 wherein the gene encoding protein X is isolated from a diol dehydratase gene cluster.

37. The microorganism of Claim 35 wherein the glycerol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella* and *Citrobacter*.

38. The microorganism of Claim 34 wherein the diol dehydratase gene cluster is from an organism selected from the genera consisting of *Klebsiella*, *Clostridium* and *Salmonella*.

39. The method of Claim 32 wherein the microorganism is selected from the group consisting of *Citrobacter*, *Enterobacter*, *Clostridium*, *Klebsiella*, *Aerobacter*, *Lactobacillus*, *Aspergillus*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Debaryomyces*, *Mucor*, *Torulopsis*, *Methylobacter*, *Escherichia*, *Salmonella*, *Bacillus*, *Streptomyces* and *Pseudomonas*.

40. The method of Claim 32 further comprising the step of introducing a gene encoding at least one of protein 1, protein 2 and protein 3 into said microorganism.



	M	S	L	S	P	G	V	H	L	F	Y	D	S	R	G	Q	G	A	G	A	L	D	E	L	C	W	G	L	E	E	Q	G	V	P	C	Q	A	I	T	Majority	
	10																																								
	20																																								
	30																																								
	40																																								
	1	M	S	L	S	P	G	V	H	L	F	Y	H	S	R	W	Q	G	T	R	V	L	D	E	L	C	W	G	L	E	E	Q	G	V	P	C	R	A	I	C	cfu_orfX.aa
	1	M	S	L	S	P	G	V	R	L	F	Y	D	P	R	G	H	H	A	G	A	I	N	E	L	C	W	G	L	E	E	Q	G	V	P	C	Q	T	I	T	kpn_orfX.aa
		50																																							
		Y	D	G	G	D	A	A	L	G	A	L	A	K	S	S	T	L	R	V	G	L	G	L	S	A	S	G	D	I	A	L	T	H	A	Q	L	P	Majority		
		60																																							
		70																																							
		80																																							
	41	C	D	H	D	C	A	L	A	L	G	K	L	A	A	K	S	S	T	L	R	V	G	L	G	L	N	A	T	G	D	I	A	L	T	H	A	Q	L	P	cfu_orfX.aa
	41	Y	D	G	G	D	A	A	L	G	A	L	A	A	R	S	S	P	L	R	V	G	I	G	L	S	A	S	G	E	I	A	L	T	H	A	Q	L	P	kpn_orfX.aa	
		90																																							
		A	D	A	A	L	A	T	G	H	V	T	A	G	T	A	Q	L	R	T	L	G	A	N	A	G	Q	L	V	K	V	L	P	L	S	E	R	I	K	Majority	
		100																																							
		110																																							
		120																																							
	81	E	D	R	A	L	V	C	G	H	T	R	A	G	T	A	Q	I	R	T	L	G	A	N	A	G	Q	L	V	K	V	L	P	F	S	E	-	I	K	cfu_orfX.aa	
	81	A	D	A	P	L	A	T	G	H	V	T	D	S	D	D	Q	L	R	T	L	G	A	N	A	G	Q	L	V	K	V	L	P	L	S	E	R	N	.	kpn_orfX.aa	

FIG. 4

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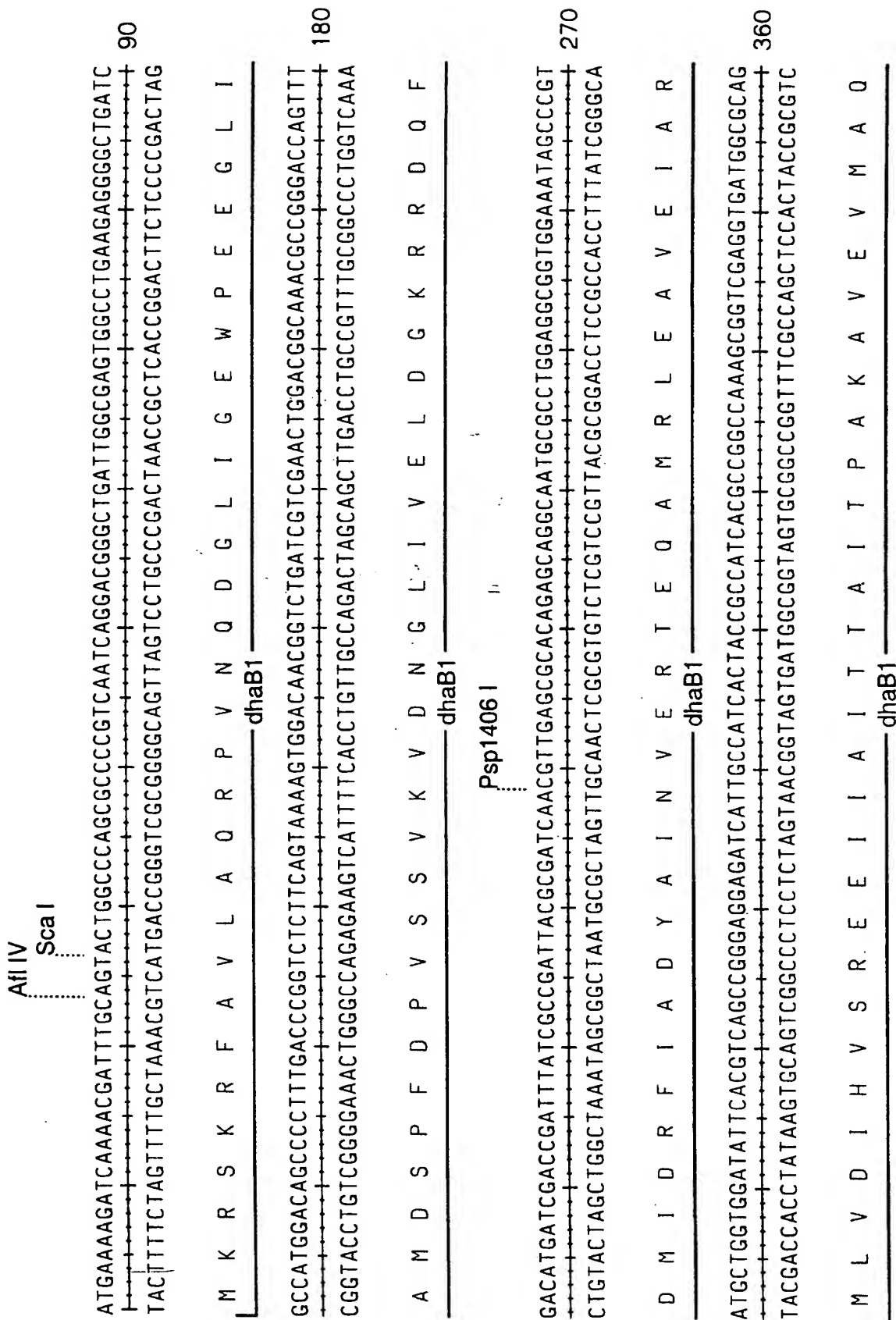


FIG.-2A-1

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ATGAACGTGGAGATGATGGCGCTGCAGAAGATGCGTGCCCGCGGACCCCTCCAACACAGTGCCACGTCACCAATCTCAAAGAT	450
TACTTGCACCACTCTACTACTACCGGACGCTCTTCTACGCACGGCGGCTGGGGAGGTTGGTCACGGTGCAGTGGTTAGAGTTTCTA	
M N V V E M M A L O K M R A R R T P S N Q C H V T N L K D	
-----dhaB1-----	
AATCCGGTGCAGATTGCCGCTGACGCCCGGAGCGCGGATCCGGGCTTCTCAGAACAGGAGACCACGGTCGGTATCGCGCGCTACGCG	540
TTAGGCCACGTCTAACGGCGACTGGCGGCTCCGGCCCTAGGCGCCGAAGAGTCTTGTCCTCTGGTGCCAGCCATAGCGCGCGGATGCGC	
N P V Q I A A D A A E A G I R G F S E Q E T T V G I A R Y A	
-----dhaB1-----	
<div> CciNI Not I </div> <div> Bsb I </div>	
CCGTTTAACGCCCTGGCGCTGTGGTCGGTTCGCAGTGCAGCGCCCGCGCGCTGTGACGCAGTGCTCGGTGGAAGAGGCCACCGAGCTG	630
GGCAAATTGCGGGACCGCGACAACCAAGCGTCACGCCGGCGGCGCCGACAACTGCGTCACGAGCCACCTTCICCGGTGGCTCGAC	
P F N A L A L L V G S Q C G R P G V L T Q C S V E E A T E L	
-----dhaB1-----	
<div> Dsa VI Acc I Fbl </div>	
GAGCTGGGCATGCGTGGCTTAACCAGCTACGCCGAGACGGTGTGGTCTACGGCACCGAAGCGGTATTTACCGACGGCGATGATACGCCG	720
CTGACCCCGTACGCACCGAAATTGGTCGATGCGGCTCTGCCACAGCCAGATGCCGTGGCTTCGCCATAAATGGCTGCCGCTACTATGCGGC	
E L G M R G L T S Y A E T V S V Y G T E A V F T D G D D T P	
-----dhaB1-----	

FIG..2A-2

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Eco47 III
TGGTCAAAGGCGTTCCCTCGCCTACGCCCTCCCGGGGTGAAATGCGCTACACCICCGGCACCGGATCCGAAGCGCTGATGGGC
810
ACCAAGTTCCGCAAGGAGCGGAGCCGGATGCGGAGGGCGCCCAACTTTACGCGATGTGGAGGCCGTGGCCTAGGCTTCGCGGACTACCCG

W S K A F L A S A Y A S R G L K M R Y T S G T G S E A L M G
dhaB1

EcoE I Bsa XI
TATTCGGAGAGCAAGTCGATGCTCTACCTCGAATCGCGCTGCATCTTCATTAAAGGCGCGGGTTCAGGGACTGCAAAACGGCGCG
900
ATAAGCCTCTCGTTCAGTACGAGATGGAGCTTAGCGCGACGTAGAGTAATGATTTCCGCGGCCCAAGTCCCTGACGTTTTTGCCCGCGC

Y S E S K S M L Y L E S R C I F I T K G A G V Q G L Q N G A
dhaB1

GTGAGCTGTATCGGCATGACCGGCGCTGTGCCGTGGGCAATTCGGGCGGTGCTGGCGGAAACCTGATCGCCTCTATGCTCGACCTCGAA
990
CACTCGACATAGCCGTACTGGCCGCGACACGGCAGCCCGTAAGCCCGCCACGACCGCCTTTTGGACTAGCGGAGATACGAGCTGGAGCTT

V S C I G M T G A V P S G I R A V L A E N L I A S M L D L E
dhaB1

GTGGCGTCCGCCAACGACCAAGACTTTCCTCCACTCGGATATTGCGCGCACCGCGGCACCCCTGATGCAGATGCTGCCGGCACCGACTTT
1080
CACCAGGCGGTTGCTGCTGAAAGAGGGTGAGCCTATAAGCGGCGTGGCGCGGTGGGACTACGCTACGACGGCCCGGCTGGCTGAAA

V A S A N D O T F S H S D I R R T A R T L M Q M L P G T D F
dhaB1

FIG. 2B-1

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Pfl1108 I

ATTTCTCCGGCTACAGCGGGTGCCGAACACGACAAACATGTTCCCGGCTCGAACTTCGATCGGAAGATTTTGATGATTACAACATC
 TAAAGAGGCCGATGTCGCCCCACGGCTTGATGCTGTTGTACAAGCGGCCGAGCTTGAAGCTACGCCCTTCTAAAACTACTAATGTGTGAG
 1170

I F S G Y S A V P N Y D N M F A G S N F D A E D F D D Y N I
 dhaB1

CTGCAGCGTGACCTGATGGTTGACGGGGCCCTGCGTCCGGTGACCGAGGGCGGAACCATTTGCCATTGCCAGAAAGCGGCGCGGCGATC
 GACGTGCACTGGACTACCAACTGCCCGCGGACGCGAGGCCACTGGCTCCGCCCTTGGTAACGGTAAGCGGTCTTTCGCCGCGCCCGCTAG
 1260

L Q R D L M V D G G L R P V T E A E T I A I R Q K A A R A I
 dhaB1

CAGGCGGTTTTCGCGAGCTGGGGCTGCCGCAATCGCCGACGAGGAGGTGGAGGCCGCCACCTACGCGCACGGCAGCAACGAGATGCCG
 GTCCGCCAAAAGGCGCTCGACCCCGACGGCGGTTAGCGGCTGCTCCTCCACCTCCGGCGGTGGATGCGGTGCCGTGCTGTCTACGGC
 1350

Q A V F R E L G L P P I A D E E V E A A T Y A H G S N E M P
 dhaB1

CCGCGTAACGTGGAGGATCTGAGTGGGTGGAAGAGATGATGAAGCGCAACATCACCAGCCCTCGATATTGTCGGCGCGCTGAGCCGC
 GGCGCATTGCACCACTCCTAGACTCAGCCACCTTCTCTACTACTTCCGCTTGTAGTGGCCGGAGCTATAACAGCCCGCGGCGACTCGGCG
 1440

P R N V V E D L S A V E E M M K R N I T G L D I V G A L S R
 dhaB1

FIG. 2B-2

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AGCGGCTTTGAGGATATCGCCAGCAATATTTCTCAATATGCTGCGCCAGCGGGTCACCGGGATTAACCTGCAGACCTCGGCCATTCTCGAT
 TCGCCGAAACTCCTATAGCGGTCGTTATAAGAGTTATACGACGCGGTGCGCCAGTGGCCGCTAATGGACGCTCTGGAGCCGGTAAGAGCTA
 S G F E D I A S N I L N M L R Q R V T G D Y L O T S A I L D
 -----dhaB1----- 1530

FIG._2B-3

CGGCAGTTCGAGGTGGTGAGTGGGTCAACGACATCAATGACTATCAGGGCGGGCACCGGCTATCGCATCTCTGCCGAACGCTGGGCG
 GCCGTCAAGCTCCACCACTCAGCCAGTTGCTGTAGTTACTGATAGTCCCGCGCCCGTGGCCGATAGCGTAGAGACGGCTTGGACCCCGC
 R O F E V V S A V N D I N D Y Q G P G T G Y R I S A E R W A
 -----dhaB1----- 1620

Drd II

GAGATCAAAAATATTCGGGCGGTGGTTCAGCCCGACACCATTTGAATAAGGCGGTATTCCTGTGCAACAGACAACCCAAATTCAGCCCTCT
 CTCTAGTTTTTATAAGGCCCGCACCAAGTCCGGCTGTGGTAACCTATTCCGCCATAAGGACACGTTGCTCTGTGGTTTAAGTCGGGAGA
 E I K N I P G V V Q P D T I E
 -----dhaB1----- 1710

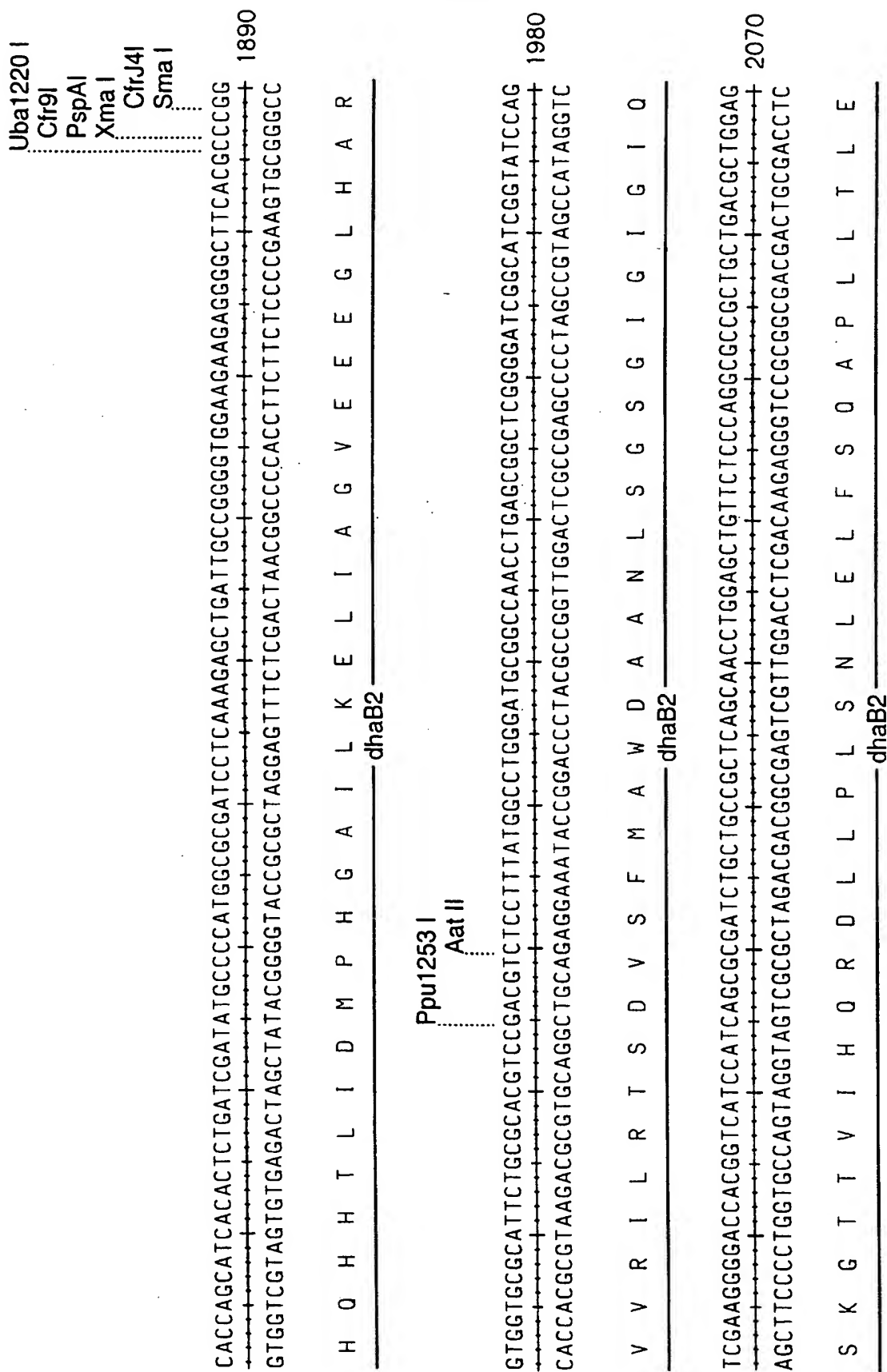
H_{dh}

V Q Q T T O I O P S
 -----dhaB2-----
 TTTACCTGAAACCCGAGGGCGGGTAGCTTCTGCCGATGAACGCGCGCATGAAGTGGTGATCGGCGTCGGCCCTGCCTTCGATAAA
 AAATGGGACTTTTGGGCGCTCCCGCCCATCGAAGACGGCTACTTGGCGGGTACTTCACCACTAGCCGACGCCGGGACGGAAGCTATTT
 F T L K T R E G G V A S A D E R A D E V V I G V G P A F D K
 -----dhaB2----- 1800

FIG._2C-1

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**FIG._2C-2**

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ACCTACCGGCAGATTGGCAAAACGCTGCGGCTATGCGCGCAAGAGTCACCTTCGCCGGTGCCGGTGGTGAACGATCAGATGGTGCGG 2160
 TGGATGGCCGCTCTAACCGTTTTTGGCAGCGCGGATACGCGGTTTCTCAGTGAAGCGGCCACGGCCACCACATTGCTAGTCTACCACGCC
 T Y R Q I G K N A A R Y A R K E S P S P V V N D Q M V R
 —dhaB2—

FIG._2C-3

CCGAAATTTATGGCCAAAGCCGCGCTATTTATATCAAGAGACCAAAACATGTTGGTGCAGGACGCCGAGCCCGTCACCCCTGCACATCGAC 2250
 GGCTTTAAATACCGGTTTCGGCGCGGATAAAGTATAGTTTCTCTGGTTGTACACCACGTCCTCGGGCTCGGGCAGTGGGACGTGTAGCTG
 P K F M A K A A L F H I K E T K H V V O D A E P V T L H I D
 —dhaB2—

TTAGTAAGGGAGTGACCATGAGCGAGAAAACCATGCGCGTGCAGGATTATCCGTTAGCCACCCGCTGCCCGGAGCATATCCTGACGCCTA 2340
 AATCATCCCTCACTGGTACTCGCTCTTTTGGTACGCGCACGTCCTAATAGGCAATCGGTGGGCGACGGGCCCTCGTATAGGACTGCGGGAT
 M S E K T M R V Q D Y P L A T R C P E H I L T P
 —dhaB3—

L V R E
 —dhaB2—

FIG._2D-1

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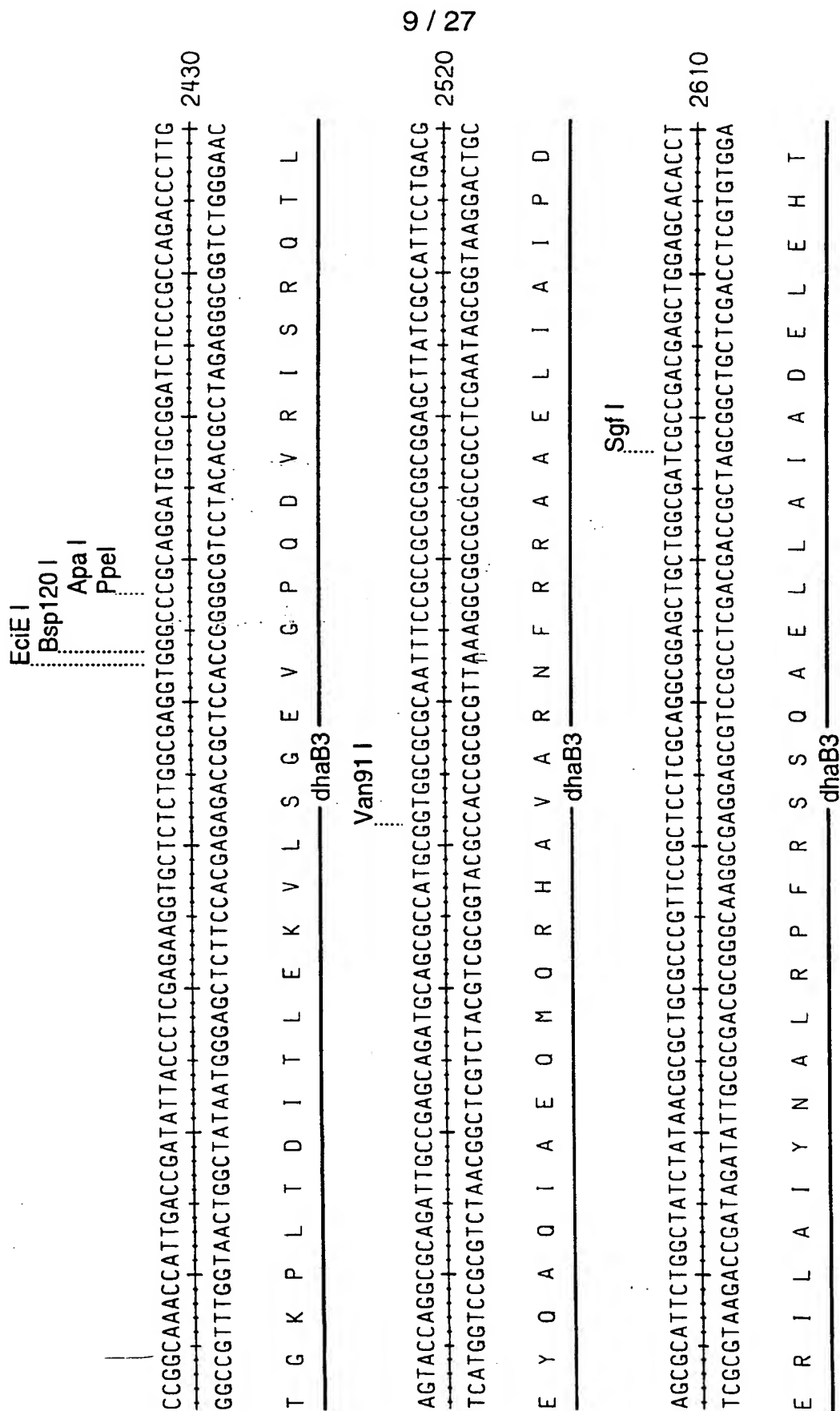


FIG. 2D-2

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GGCATGCGACAGTGAATGCGGCTTTGTCCGGGAGTCGGGGAAGTGTATCAGCAGCGGCGATAGCTGCGTAAAGGAAGCTAAGCGGAGG 2700
 CCGTACGCTGTCACTTACGGCGGAAACAGGCCCTCAGCGCCTTACATAGTCGTGCGCGTATTCGACGCATTTCCCTTCGATTGCGCTCC
 W H A T V N A A F V R E S A E V Y Q O R H K L R K G S
 dhaB3

XcmI

TCAGCATGCCGTTAATAGCCGGGATTGATATCGGCAACGCCACCACCGAGGTGGCGCTGGCGTCCGACTACCCGCGAGGCGGCGTTTG 2790
 AGTCGTACGGCAATTATCGGCCCTAACTATAGCCGTTGCGGTGGCTCCACCAGCGACCGCAGGCTGATGGGCGTCCGCTCCCGCAAAC
 M P L I A G I D I G N A T T E V A L A S D Y P O A R A F
 dhaB4

FIG._2D-3

SanD I

TTGCCAGCGGGATCGTCGCGACGACGGGCATGAAAGGGACGCGGGACAATATCGCCGGGACCCCTCGCCGCGCTGGAGCAGGCCCTGGCGGA 2880
 AACGGTCGCCCTAGCAGCGCTGCTGCCCGTACTTTCCCTGCGCCCTGTTATAGCGGCCCTGGGAGCGCGCGACCTCGTCCGGGACCGCT
 V A S G I V A T T G M K G T R D N I A G T L A A L E O A L A
 dhaB4

AAACACCGTGGTCGATGAGCGATGTCCTCGCATCTATCTTAACGAAGCCGCGCGGTGATGGCGAIGTGGCGATGGAGACCAATCACCG 2970
 TTTGTGGCACCACTACTCGCTACAGAGAGCGTAGATAGAAATGCTTCGGCGCGGCCACTAACCGCTACACCGCTACCTCTGGTAGTGGC
 K T P W S M S D V S R I Y L N E A A P V I G D V A M E T I T
 dhaB4

FIG._2E-1

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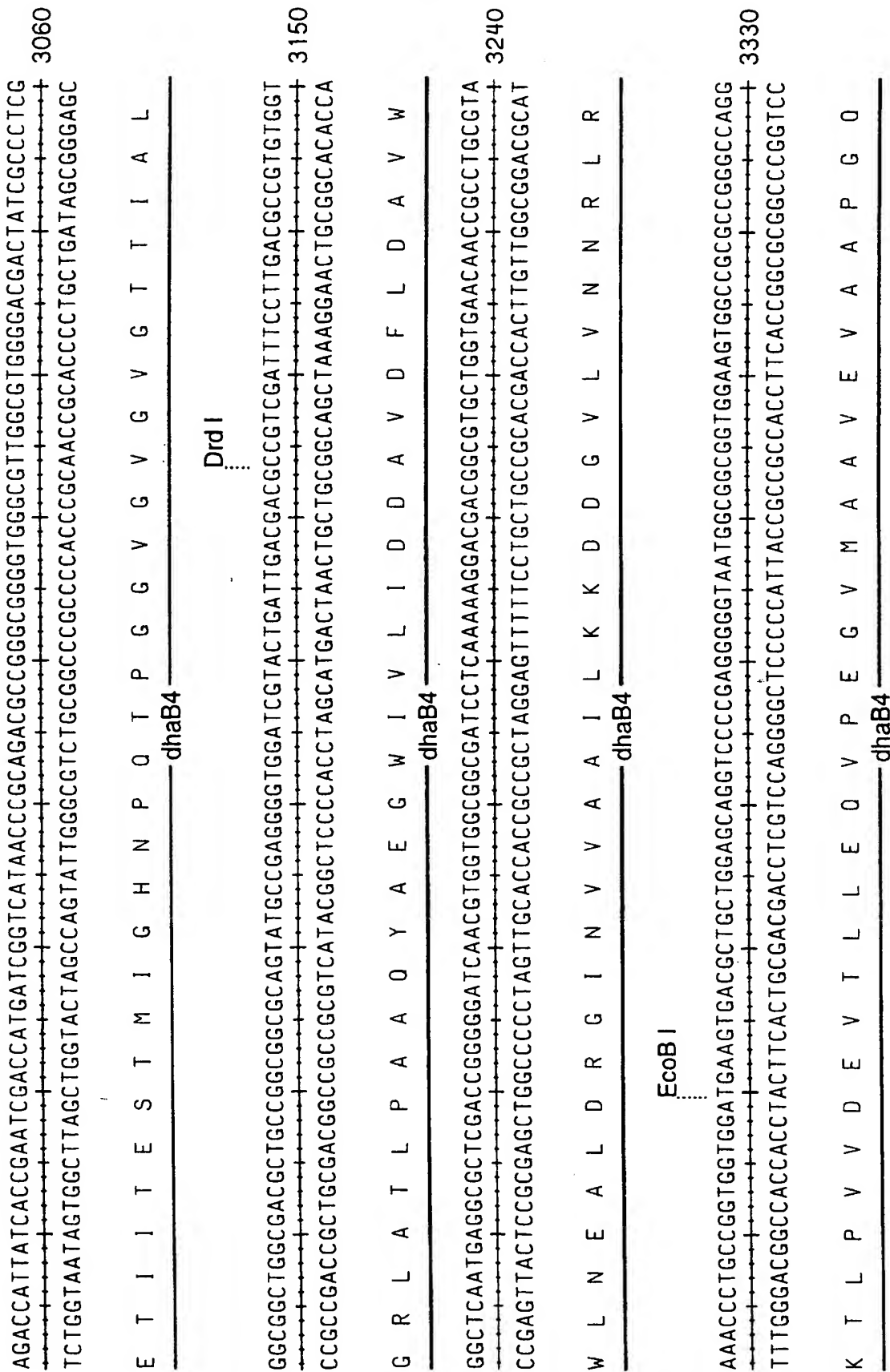


FIG..2E-2

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TGGTGGGATCCTGTGGAATCCCTACGGGATCGCCACCTTCTTCGGGCTAAGCCCGGAAGAGACCCAGGCCATCGTCCCCATCGCCCCGCG 3420
 ACCACGCCTAGGACAGCTTAGGGATGCCCTAGCGGTGGAAGAGCCCGATTTCGGGCCCTTCTCTGGGTCCGGTAGCAGGGGTAGCGGGCGCG
 V V R I L S N P Y G I A T F F G L S P E E T O A I V P I A R
 -----dhaB4-----
 CCTTGATTGGCAACCGTTCGCGGGTGGTGCTCAAGACCCCGCAGGGGGATGTGCAGTCGCGGGTGATCCCGCGGGCAACCTCTACATTA 3510
 GGGACTAACCGTTGGCAAGGCGCCACCACGAGTTCTGGGGCGTCCCCCTACACGTCAGCGCCCACTAGGGCCGCCCGTTGGAGATGTAAT
 A L I G N R S A V V L K T P O G D V O S R V I P A G N L Y I
 -----dhaB4-----
 GCGCGAAAGCGCGGAGAGGCCGATGTGCGCGAGGGCGCGGAAGCCATCATGCAGGCGATGAGCGCTCGCGTCCGGTACGCGACA 3600
 CGCGCTTTTCGCGGCGCCTCTCCGGCTACAGCGGCTCCCGCGCCTTCGGTAGTACGTCCGCTACTCGCGGACGCGAGGCCCATGCGGCTGT
 S G E K R R G E A D V A E G A E A I M O A M S A C A P V R D
 -----dhaB4-----

FIG._2E-3

Taq II'

TCCGCGCGGAACCGGCGCACCCACGCGCGGCGCATGCTTGAGCGGGTGGCAAGGTATGGCGTCCCTGACCGGCCCATGAGATGAGCGCGA 3690
 AGCGCGCGCTTGCGCCGCTGGGTGCGGCGCGCGGTACGAACTCGCCCCACGCGTTCCATTACCGCAGGGACTGGCCCGTACTCTACTCGCGCT
 I R G E P G T H A G G M L E R V R K V M A S L T G H E M S A
 -----dhaB4-----

FIG._2F-1

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TATACATCCAGGATCTGCTGGCGGTGGATACGTTTATTCGCGCAAGGTGCAGGGCGGGATGGCCGGCGAGTGCGCCATGGAGAATGCCG 3780
 ATATGTAGTCTTAGACGACCGCCACCTATGCAAAATAGGGCGGTTCACGTCCCGCCCTACCGGGCGCTCAGCGGTACCTCTTACGGC
 I Y I Q D L L A V D T F I P R K V Q G G M A G E C A M E N A
 -----dhaB4-----
 TCGGGATGGCGCGGATGGTGAAAGCGGATCGTCTGCAATGCAGGTATCGCCCGCAACTGAGCGCCGACTGCAGACCGAGGTGGTGG 3870
 AGCCCTACCGCGCTACCACTTTCGCCTAGCAGACGTTTACGTCCAATAGCGGGCGCTTGACTCGCGGGCTGACGTCTGGCTCCACCACC
 V G M A A M V K A D R L Q M Q V I A R E L S A R L Q T E V V
 -----dhaB4-----
 TGGGCGCGTGGAGGCCAACATGGCCATCGCCGGGGCGTTAACCACTCCGGCTGTGCGGGCGCGCTGGCGATCCTCGACCTCGGCGCGG 3960
 ACCCGCCGCACCTCCGGTTGTACCGGTAGCGGGCCCCCGCAATTGGTGAGGGCCGACACGCCCGCGGCGACCGCTAGGAGCTGGAGCGCGCGG
 V G G V E A N M A I A G A L T T P G C A A P L A I L D L G A
 -----dhaB4-----
 GCTCGACGGATCGCGCGATCGTCAACGCGGAGGGGCAGATAACGGCGGTCCATCTCGCCGGGGGGAATATGGTCAGCCCTGTTGATTA 4050
 CGAGCTGCCCTACGCCGCTAGCAGTTGGCCCTCCCGGCTCCTGCGGCTAGAGCGGGCCCCCGCCCTTATACCAGTCGGACAACATAAT
 G S T D A A I V N A E G Q I T A V H L A G A G N M V S L L I
 -----dhaB4-----

SdiI
 SfiI
 BsaKI
 BstHPI
 HpaI

FIG. 2F-2

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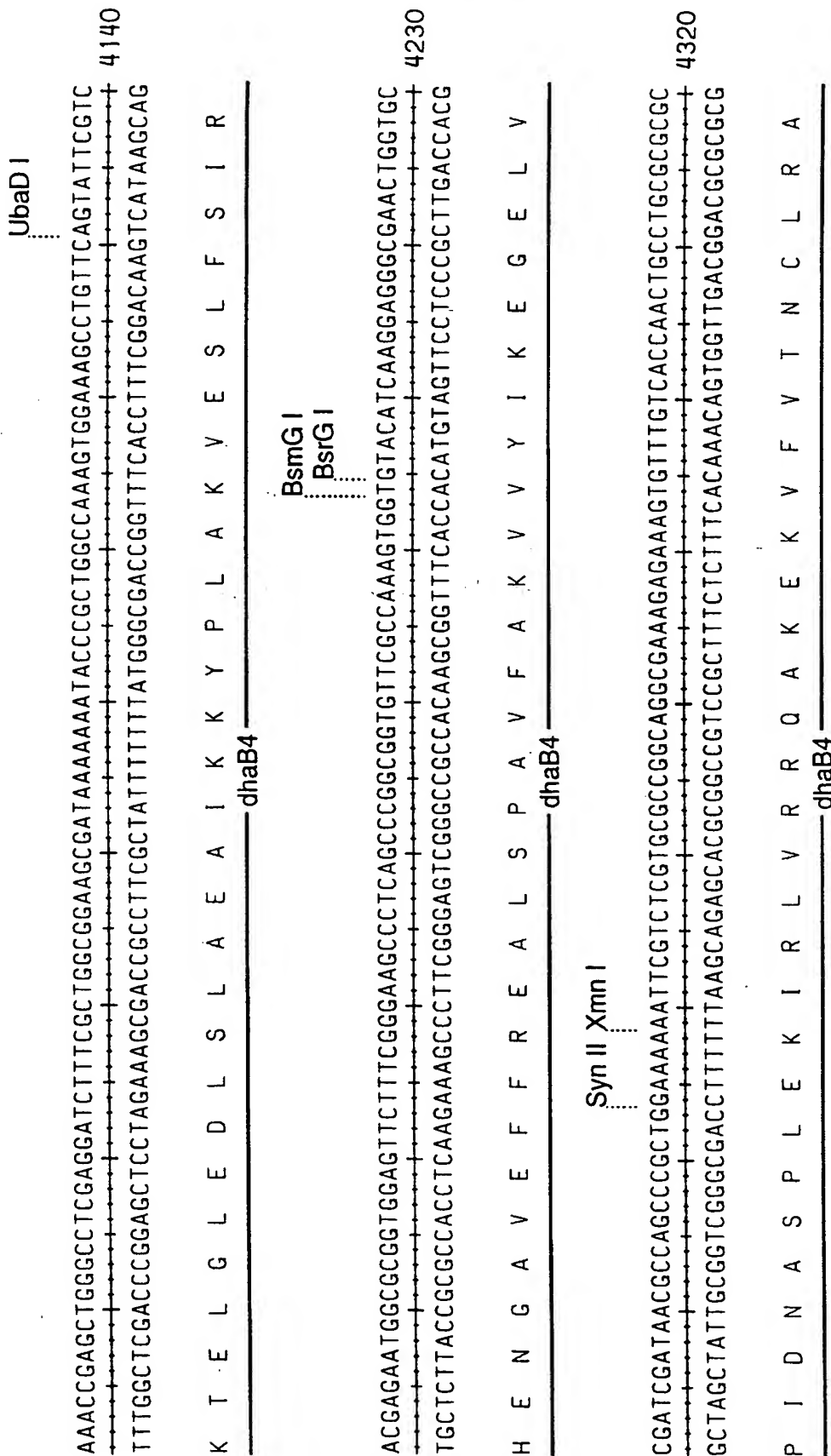
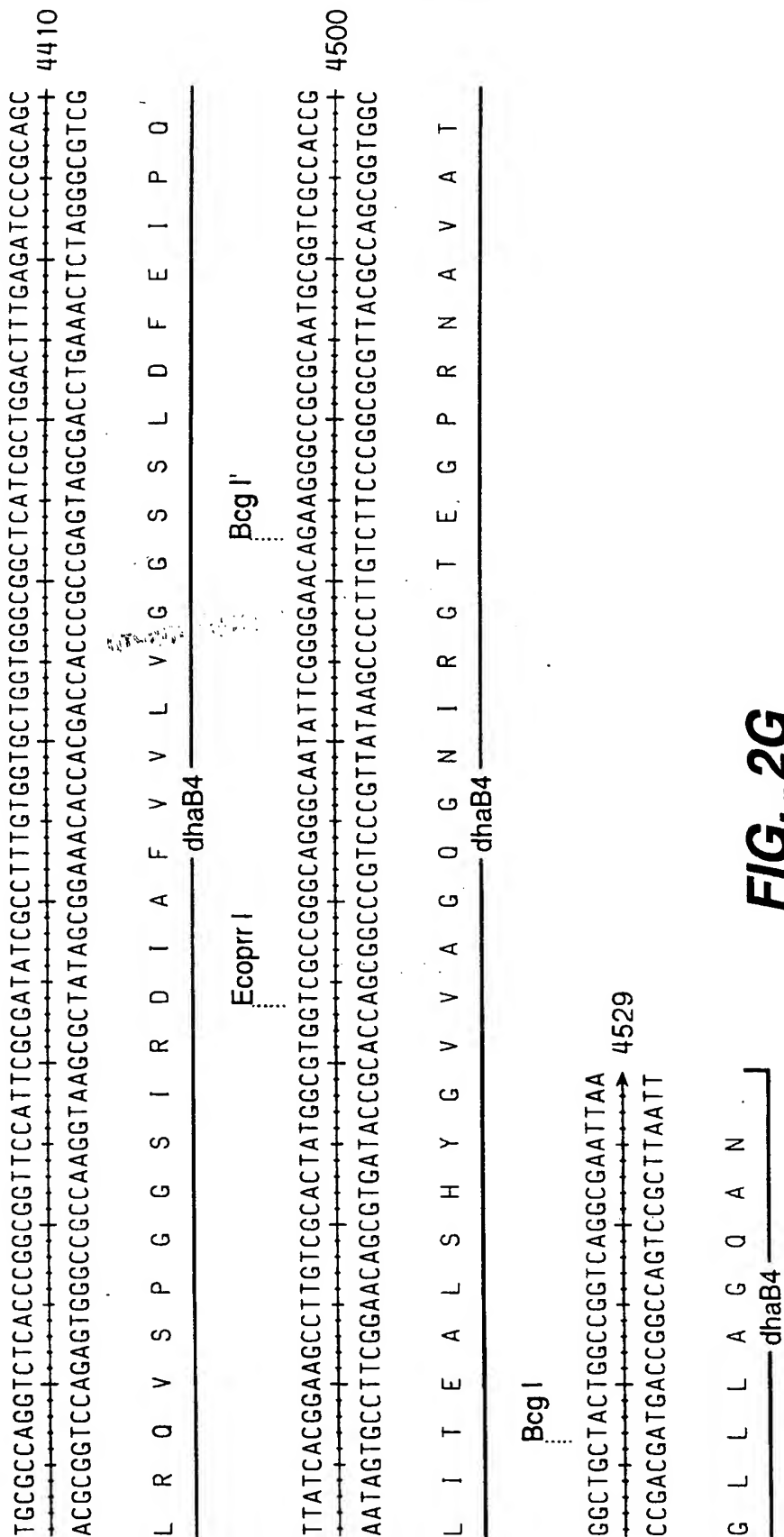


FIG. 2F-3

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	M	M	N	K	S	Q	Q	V	A	T	I	T	L	A	A	Q	Q	M	A	A	V	E	A	K	A	L	E	I	N	V	A	V	F	S	V	D	Majority				
1	M	-	N	K	S	Q	Q	I	A	T	I	T	L	A	A	A	K	K	M	A	Q	A	V	E	A	K	A	L	E	I	N	V	P	V	F	S	V	D	cfu_orfY.aa		
1	M	M	N	K	S	Q	Q	V	Q	T	I	T	L	A	A	Q	Q	M	A	A	V	E	K	K	A	T	E	I	N	V	A	V	F	S	V	D	Kpn_orfY.aa				
	H	G	G	N	T	L	L	I	Q	R	M	D	D	A	F	V	S	S	C	D	I	S	L	N	K	A	Y	S	A	C	S	L	K	Q	G	T	H	E	I	T	Majority
40	H	G	G	N	T	L	L	M	Q	R	M	D	D	A	F	V	T	S	C	D	I	S	L	N	K	A	Y	T	A	C	C	L	R	Q	G	T	H	E	I	T	cfu_orfY.aa
41	R	G	G	N	T	L	L	I	Q	R	M	D	E	A	F	V	S	S	C	D	I	S	L	N	K	A	W	S	A	C	S	L	K	Q	G	T	H	E	I	T	Kpn_orfY.aa
	S	A	V	Q	P	G	A	S	L	Y	G	L	Q	L	T	N	Q	Q	R	I	V	I	F	G	G	G	L	P	V	I	L	N	G	Q	V	I	G	A	V	G	Majority
80	D	A	V	Q	P	G	A	S	L	Y	G	L	Q	L	T	N	Q	Q	R	I	V	I	F	G	G	G	L	P	V	I	L	N	G	K	V	I	G	A	V	G	cfu_orfY.aa
81	S	A	V	Q	P	G	Q	S	L	Y	G	L	Q	L	T	N	Q	Q	R	I	I	I	F	G	G	G	L	P	V	I	F	N	E	Q	V	I	G	A	V	G	Kpn_orfY.aa
	V	S	G	G	T	V	E	Q	D	Q	L	L	A	E	T	A	L	D	C	F	S	A	L																	Majority	
120	V	S	G	G	T	V	E	Q	D	R	L	L	A	E	T	A	L	D	C	F	S	E	L																cfu_orfY.aa		
121	V	S	G	G	T	V	E	Q	D	Q	L	L	A	Q	C	A	L	D	C	F	S	A	L																Kpn_orfY.aa		

FIG.-3

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	MYRIYTRTGDN	GT	TAL	FGGS	RID	KDD	IR	VE	AY	GT	VDEL	IS	Majority
	10		20		30		40						
1	MYRIYTRTGDN	GT	TAL	FGGS	RID	KDD	IR	VE	AY	GT	VDEL	IS	cfu_orfW.aa
1	MYRIYTRTGDK	GT	TAL	YGGS	RIE	KDH	IR	VE	AY	GT	VDEL	IS	kpn_orfW.aa
	QLGVCYASTR	DAG	LR	ESL	HA	IQ	QT	LF	VL	GA	EL	AS	DA
	50		60		70		80						
41	QLGVCYASTR	QAE	LR	QEL	HAM	QK	ML	FL	VL	GA	EL	AS	DA
41	QLGVCYATTR	DAG	LR	ESL	HH	IQ	QT	LF	VL	GA	EL	AS	DA
	90		100		110		120						
	RLSQ	TIG	EED	I	TAL	EQ	LID	RN	MA	ES	GP	LP	KE
81	RLKQ	RIG	EED	I	QAL	EQ	LID	RN	MA	Q	SG	PL	KE
81	RLSQ	TIG	EEE	IT	AL	ER	LID	RN	MA	ES	GP	LP	KE
	AQLHV	ART	LS	RR	LE	RL	LIA	M	GR	AL	T	LR	DA
	130		140		150		160						
121	AQLHV	ART	LS	RR	LE	RL	LIA	M	GR	AL	T	LR	DA
121	AQLHV	ART	LS	RR	LE	RL	LIA	M	GR	AL	T	LR	DA
	LF	S	M	A	R	I	E	E	T	P	D	A	C
	170												
161	LF	S	M	A	R	I	E	E	T	P	D	V	C
161	LF	S	M	A	R	I	E	E	T	P	D	A	C

FIG._5

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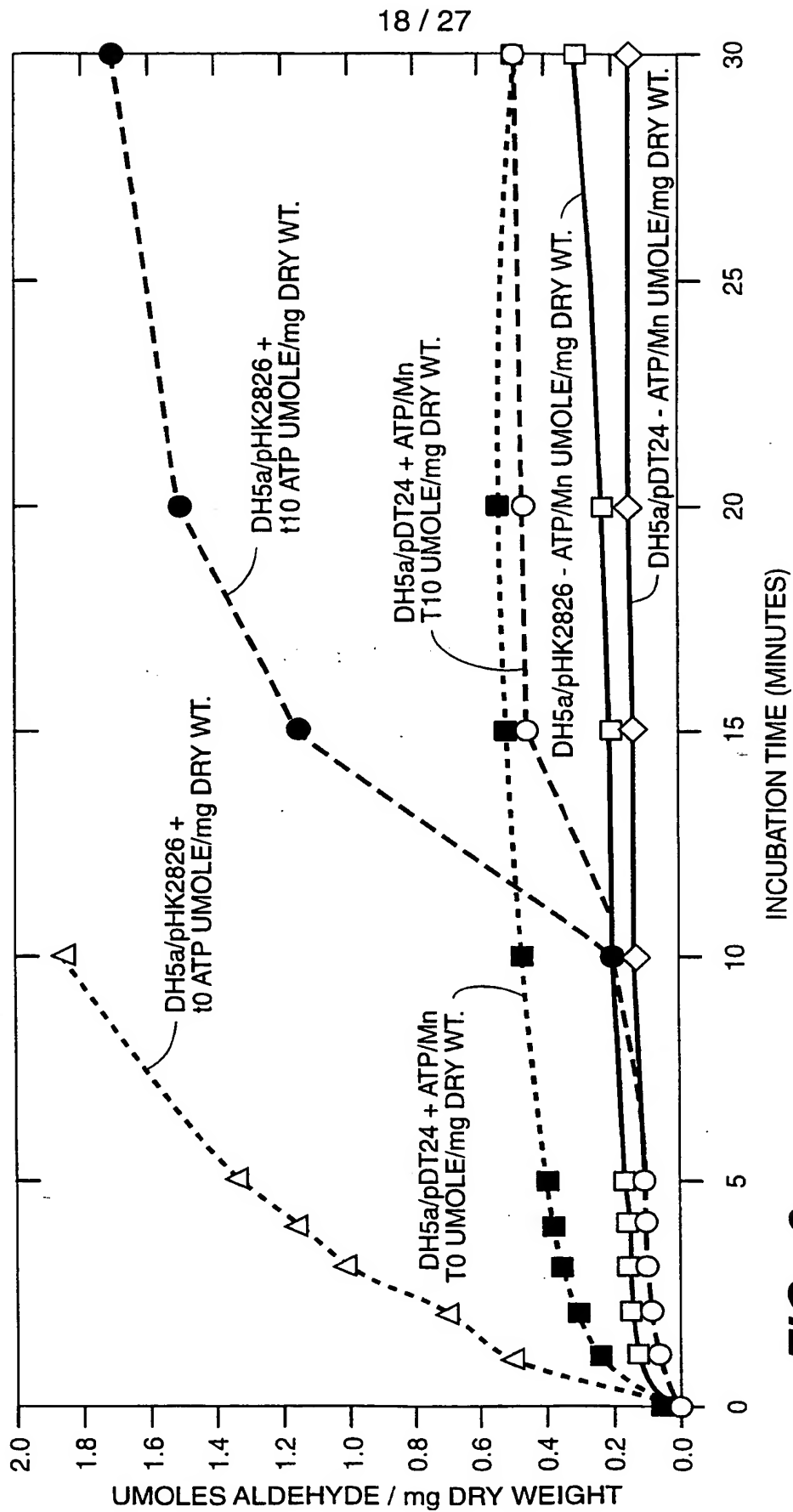


FIG. 6

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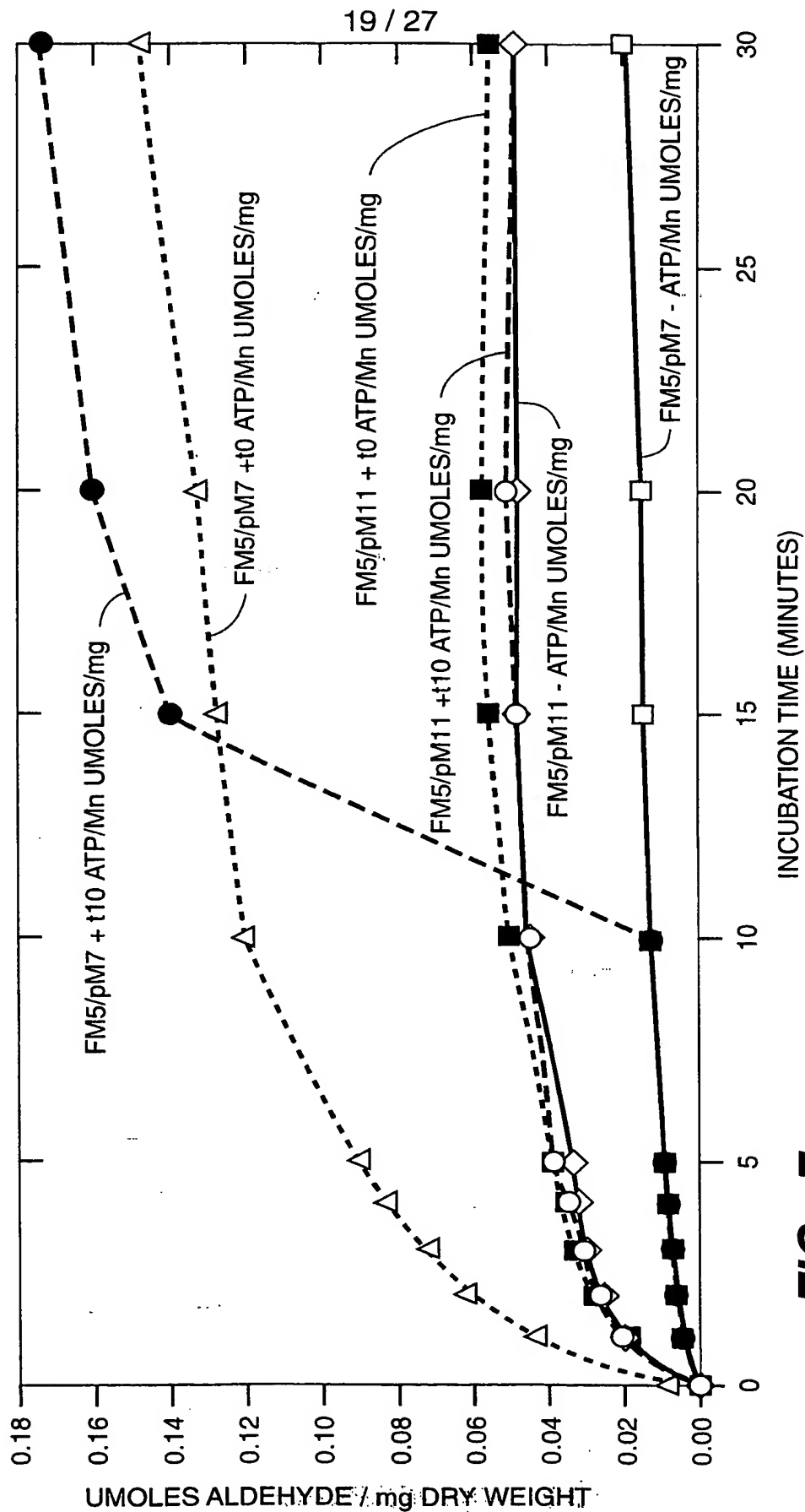


FIG. 7

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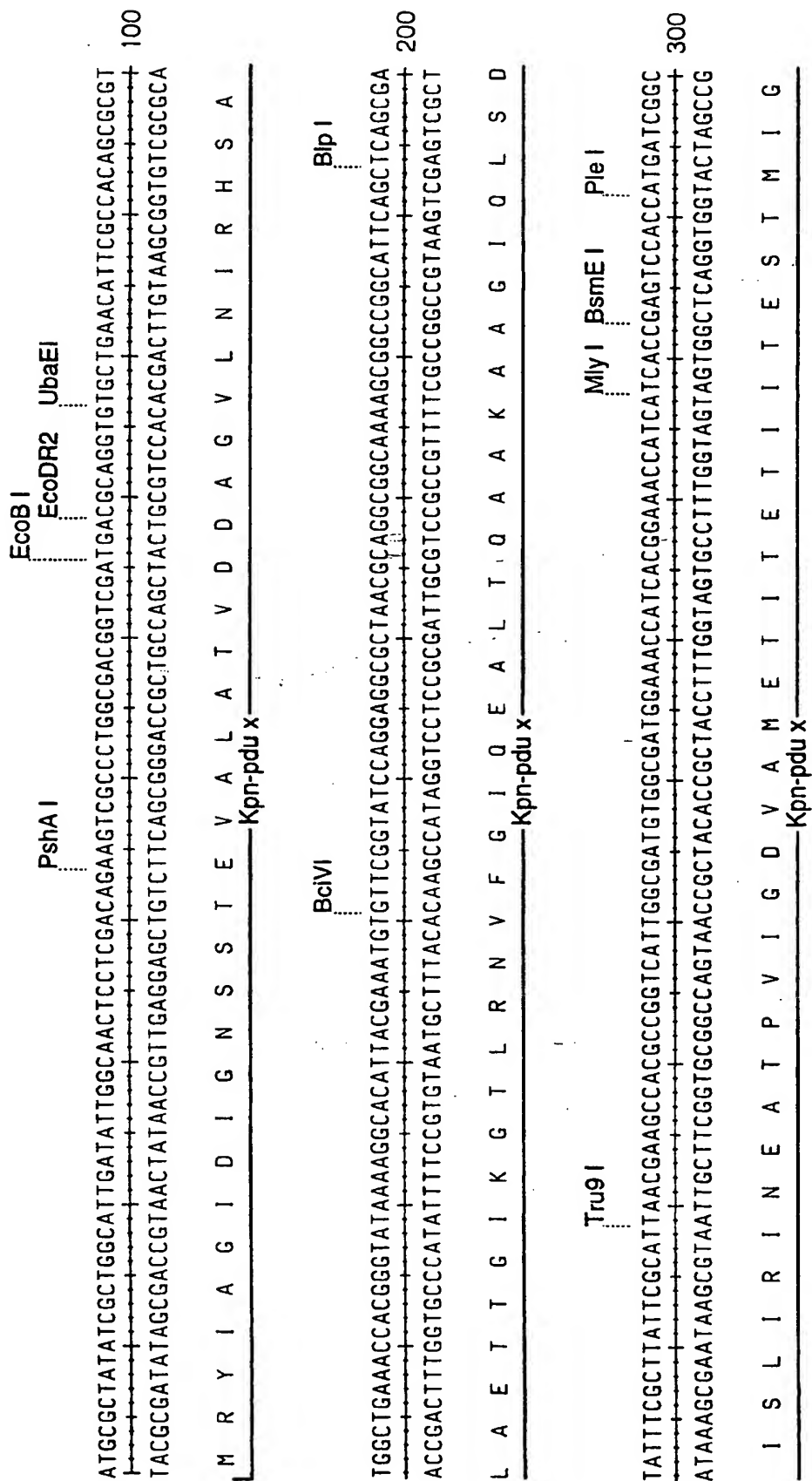


FIG._8A-1

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Bmrl Bfil StyLI
 CATAACCGAAGACACCCGGCGCTCGGACTGGGGTCCGATCACCACAGAGGCGCTGCTGCTCCGCGGACACTCCCTATATTCGG
 400
 GTATTGGGCTTCIGTGGCCGCGCAGCCTGACCCCGTAGTGGTAGTGGTCTCCGCGACGACGAGGCGCTGTGAGGGATATAAGACC
 H N P K T P G G V G L G V G I T I T P E A L L S C S A D T P Y I L
 Kpn-pdu x

FIG._8A-2

Alw26 I
 Bsa I
 BsmAI
 Bli49 I Eco31I

TGGTCTCCCTCGGCTTTGACCTTGCCGATGTGCGCGGATGGTCAATGCGGCAACGCGAGCGGCTATCAGATAACCGGCATTATTTGCGAGGAGTA
 500
 ACCAGAGGAGCCGGAAACIGAAACGGCTACAGCGGGCTACAGTTACGCCGTGCGCGGATAGTCTATTGGCCGTAATAAACGTCGTCCTACT
 V V S S A F D F A D V A A M V N A A T A A G Y Q I T G I I L Q Q D D
 Kpn-pdu x

Age I CjeP I Tfi I CjeP I'
 PinAI
 CGGCGTGTGGTCAATAACCGGTACAGCAACCGCTACCGGTGATCGACGAAGTTACGCATATCGACCGGATTCACCTTGGCATGCTGGCGGCCGTCGAG
 600
 GCCGCAGCAGCAGTTATTGGCCGATGTCGTGGCGATGGCCACTAGCTGCTTCAAGTCGTATAGCTGGCCTAAGGTGAACCGTACGACCGCCGGCAGCTC
 G V L V N N R L Q Q P L P V I D E V Q H I D R I P L G M L A A V E
 Kpn-pdu x

FIG._8B-1

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GTGCTTTACCCGGTAAGATCATCGAAACGCTCTCCAACCTTACGGTATTGCGACCGTTTCGATCTCAACGCCGAGGAGAGCCAAATATCGTGCCAA
 700
 CAGCGAAATGGGCCATTCTAGTAGCTTTGCGAGAGGTTGGGAATGCCATAACGCTGGCAAAAGCTAGAGTTGCGGCTCCCTCCTCGGTTTTATAGCACGGTT
 V A L P G K I I E T L S N P Y G I A T V F D L N A E E S Q N I V P
 Kpn-pdu x

TGGCACGGGCGCTGATTGGCAACCGCTCGGCCGTGGTGGTGAAACCCCTCCGGCGACGTCAGGCCCGGCTATTCGGCAGGTAATCTGTGTCAT
 800
 ACCGTGCCCGCGACTAACCGTTGGCGAGCCGGCACCCACTTTTGGGGAGGCCGCTGCAGTTCCGGGCGCGATAAGGCCGCTCCATTAGACAACGAGTA
 M A R A L I G N R S A V V K T P S G D V K A R A I P A G N L L L I
 Kpn-pdu x

Mbol
 BsrB I
 Ppu1253 I
 Aat II

FIG._8B-2

CGCTCAGGGGGCGCAGGTGATGTGGCCGCCGGGGGGAAGCCATCATGAAAGCGGTGACGGCTGCGGCAAACTGGACAACGTCGCGGGAGAA
 900
 GCGAGTCCCCGCGTCGCAITGTCCAACTACACCGGGGGCCCGCTTCGGTAGTACTTTCGCCAACTGCCGACGCCGTTTGACCTGTTCAGCGGCCCTCTT
 A Q G R S V Q V D V A A G A E A I M K A V D G C G K L D N V A G E
 Kpn-pdu x

Asp16H I
 Csp6 I
 CviRII
 Rsa I
 Fsu I
 Tth111 I

FIG._8C-1

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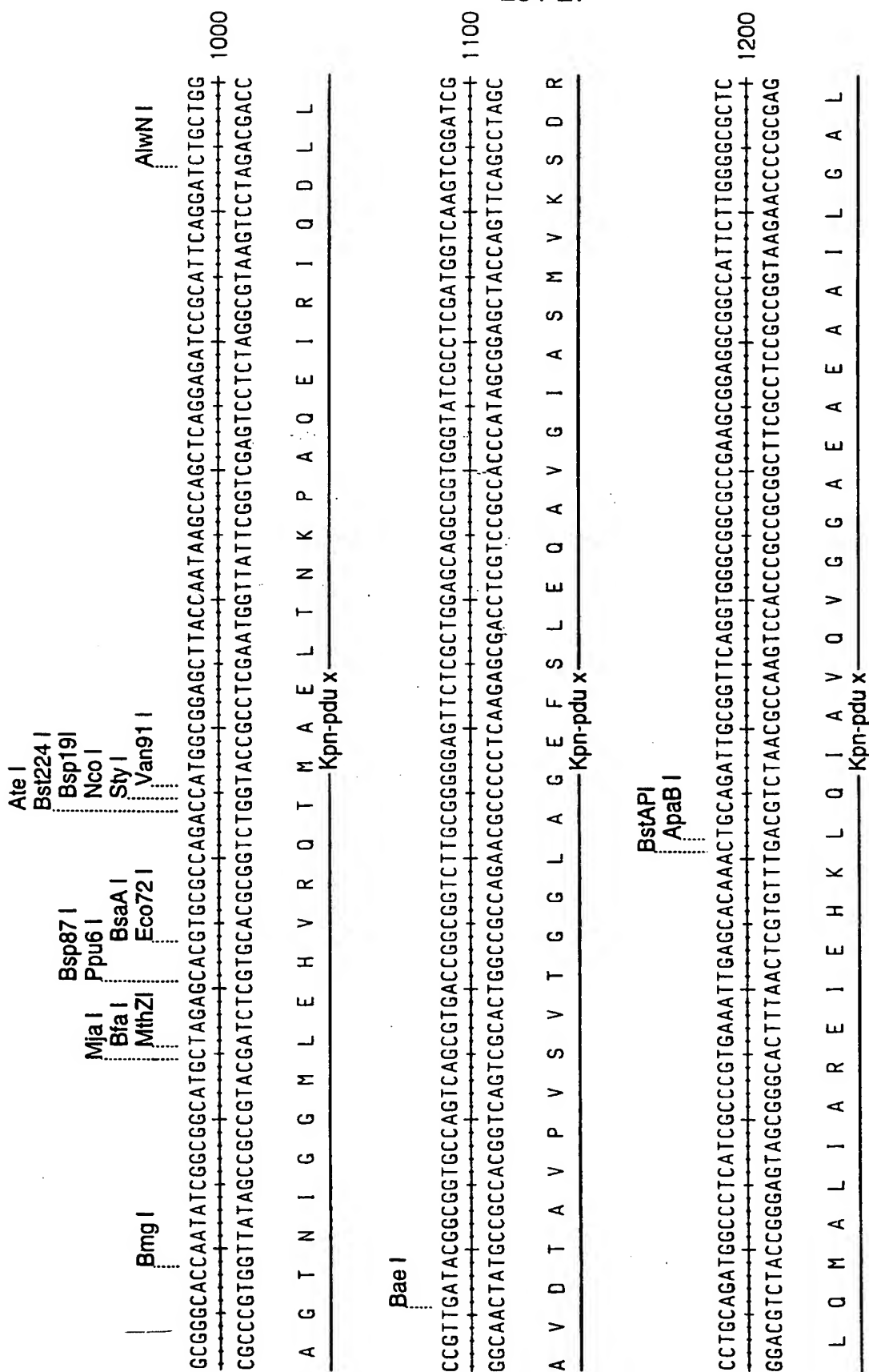


FIG._8C-2

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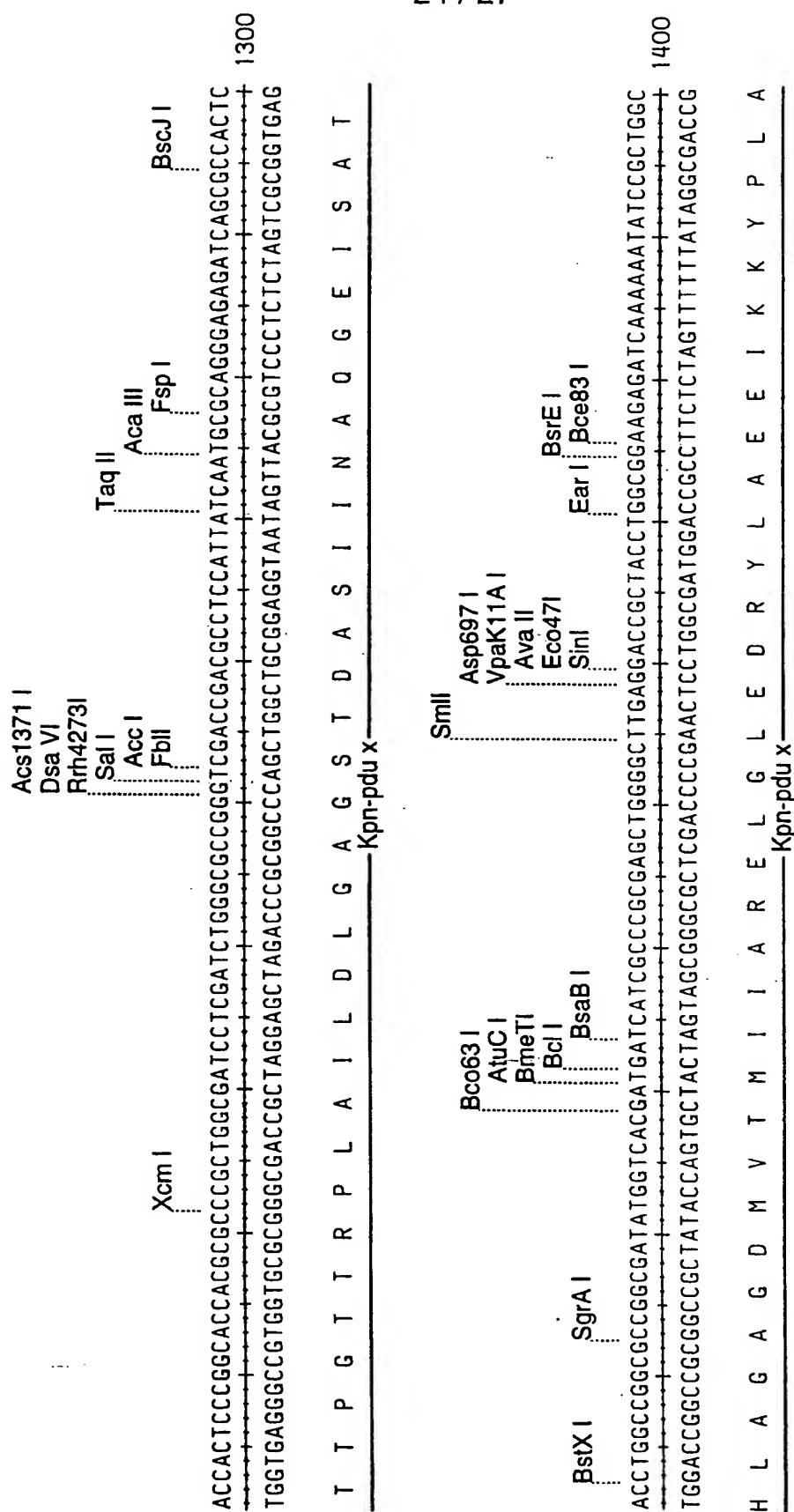


FIG. 8D-1

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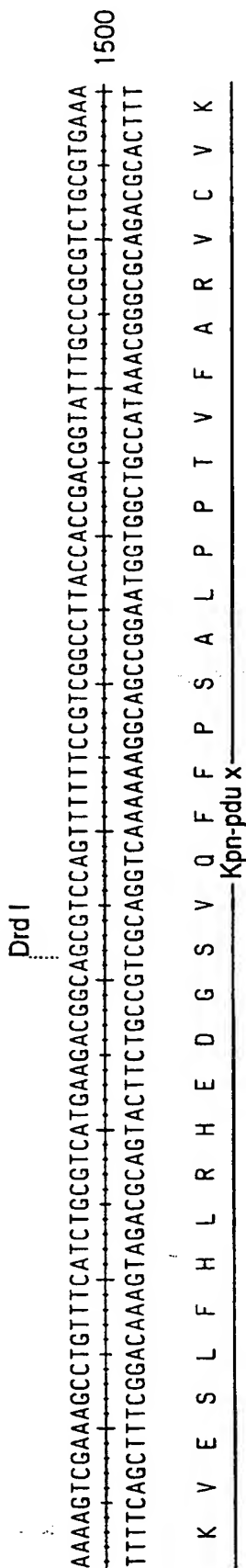


FIG._8D-2

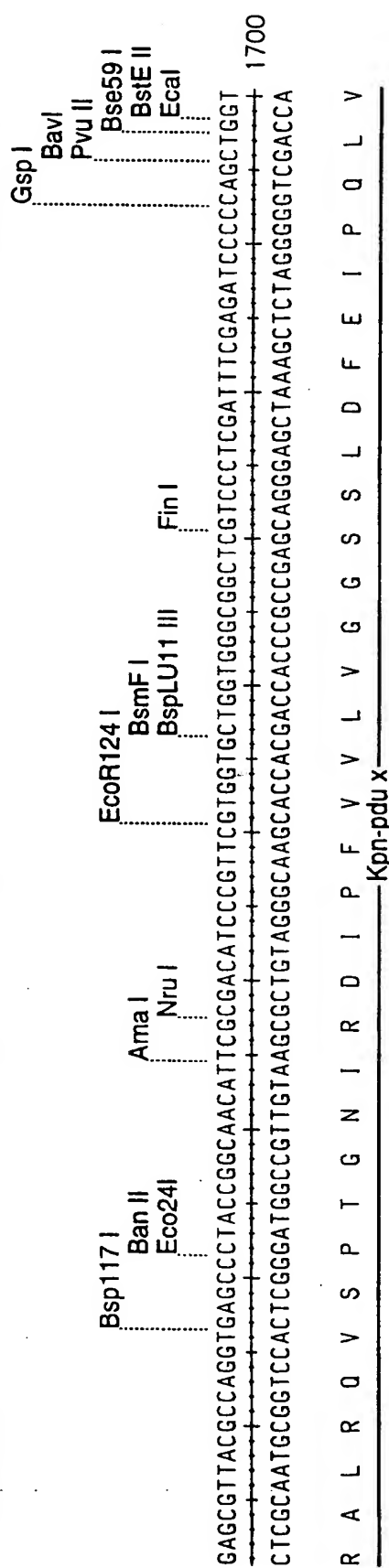
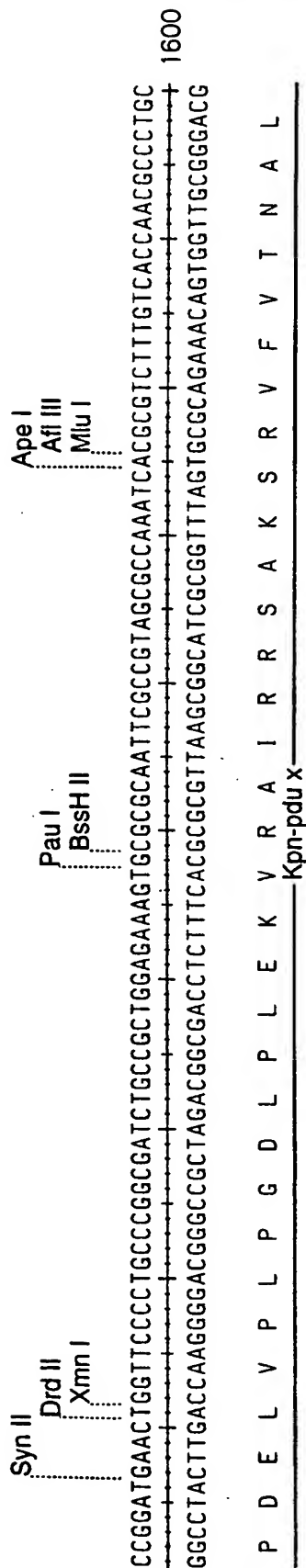


FIG._8E-1

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BseMI BsrDI
 CACCGACGGCTGGCGCACTACCGGCTGGTTGCCGGGCGGGCAACATCCGGGGCTGTGAAGGCCACGCAATGCGGTGCGCCAGCGGATTACTCCTTTCC
 GTGGCTGGCGGACCGCGTGATGGCCGACCAACGGCCCGCGCGTTGTAGGCGCCGACACTTCCGGGTGCGTTACGCCAGCGGTCGCCTAATGAGGAAAGG
 1800
 T D A L A H Y R L V A G R G N I R G C E G P R N A V A S G L L L S
 Kpn-pdu x

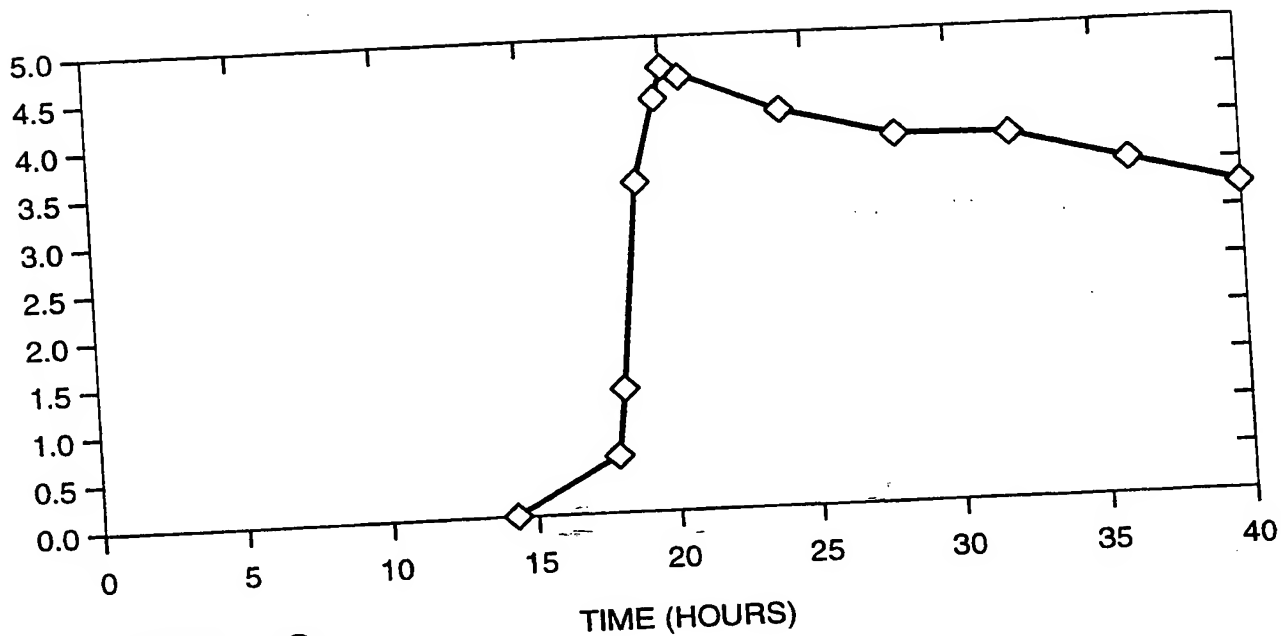
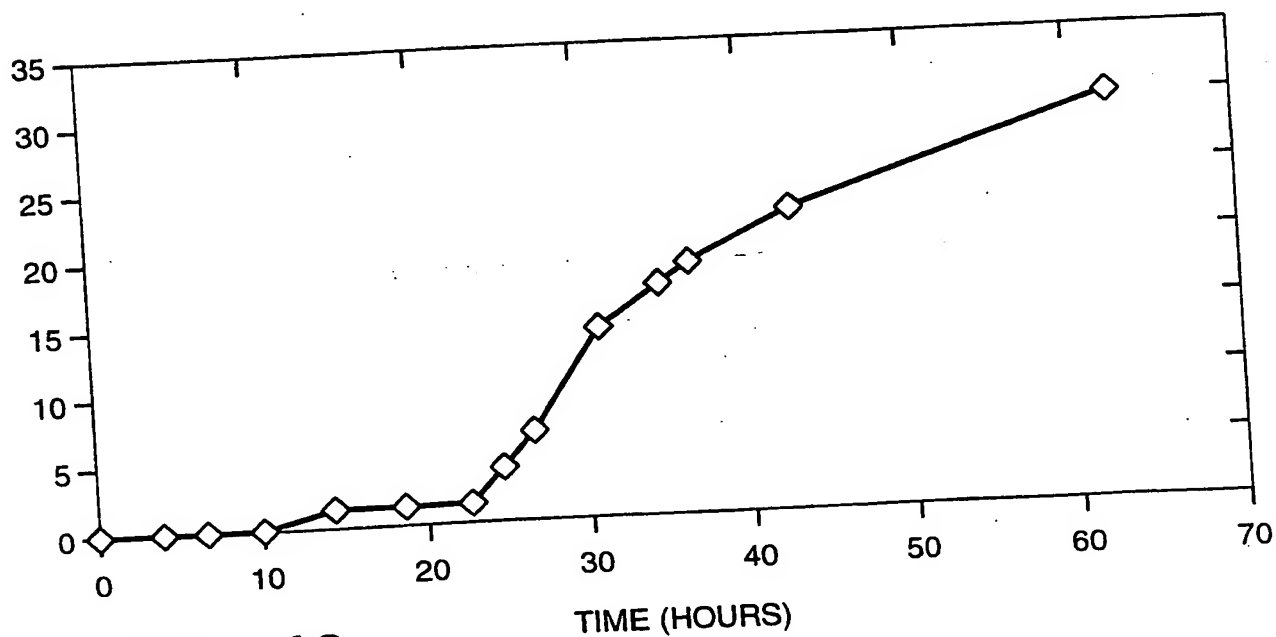
TGGCAAAAGGAGGCACACATGGAGAGTAG
 ACCGTTTTTCTCCGTGTGTACCTCTCATC
 1830

W Q K G G T H G E
 Kpn-pdu x

FIG.-8E-2

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**FIG._9****FIG._10**

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/20873

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/53 C12N15/55 C12N15/60 C12P7/18 C12N9/04
C12N9/16 C12N9/88

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X, L	WO 96 35796 A (DU PONT ; GENENCOR INT (US); LAFFEND LISA ANNE (US); NAGARAJAN VASA) 14 November 1996 see the whole document see abstract see examples 2-5 see examples 22, 23 see page 62, paragraph 2	1-40
P, A	WO 96 35795 A (DU PONT ; NAGARAJAN VASANTHA (US); NAKAMURA CHARLES EDWIN (US)) 14 November 1996 see abstract see page 9 see examples 1-3	1-13, 15-40

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

21 April 1998

Date of mailing of the international search report

08/05/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040. Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Lejeune, R

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 97/20873

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>TOBIMATSU T. ET AL.: "Molecular cloning, sequencing, and expression of the genes encoding adenosylcobalamin-dependent diol dehydrase of <i>Klebsiella oxytoca</i>." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 13, 31 March 1995, pages 7142-7148, XP002062740 cited in the application see page 7147, column 2, paragraph 2 see page 7145, column 1, line 1 - page 7146, column 1, line 4 see abstract</p>	1,5,7,8, 10,11
A	<p>TOBIMATSU T. ET AL.: "Cloning, sequencing and high level expression of the genes encoding adenosylcobalamin-dependent glycerol dehydrase of <i>Klebsiella pneumoniae</i>." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 37, 13 September 1996, pages 22352-22357, XP002057923 see abstract see page 22356, column 1, line 2 - line 5 see page 22356, column 2, paragraph 4 - page 22357, column 1, paragraph 1</p>	1,4,6,8, 10,11,15
A	<p>TONG I.T. ET AL.: "1,3-propanediol production by <i>Escherichia coli</i> expressing genes from the <i>Klebsiella pneumoniae</i> dha regulon." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 57, 1991, pages 3541-3546, XP002062741 see abstract</p>	1-4,6,8, 10,11
A	<p>SEYFRIED M. ET AL.: "Cloning, sequencing, and overexpression of the genes encoding coenzyme B12-dependent glycerol dehydratase of <i>Citrobacter freundii</i>." JOURNAL OF BACTERIOLOGY, vol. 178, no. 19, October 1996, pages 5793-5796, XP002062742 cited in the application see page 5794; figure 1 see page 5793, column 2, paragraph 5 see abstract</p>	1-4,6

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/20873

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 0826057 A	04-03-98
WO 9635795 A	14-11-96	US 5633362 A	27-05-97
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		EP 0827543 A	11-03-98

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